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CONTENTS

SECTION B—VOL. XXXIV

PAGE

No. 1—July 1951

Placentation in the Sea-Snake, *Enhydrina schistosa* (Daudin) . . . . . L. R. Kasturirangan 1

Polygalacturonase Activity of *Aspergilli* . . . . . M. S. Pavgi, H. R. Pithawala, G. R. Savur and A. Sreenivasan 33

Some Aspects of Regeneration in Leaf Cuttings of Tomato . . . . . R. S. Choudhri and V. B. Bhatnagar 43

Minor Elements in Soil and Their Effect on Forage and Feeding Cattle with Special Reference to Their Significance as Tracers of Evolution of Some Complex Organic Structures: as Determined by Application of Goldschmidt's Law . . . . . B. Rama Moorthy 46

Chromosome Evolution in Wild Populations of Acrididæ. Part I. On the Occurrence of a Supernumerary Chromosome in *Aiolopus* sp. . . . . . S. P. Ray-Chaudhuri and G. K. Manna 55

No. 2—August 1951

Additions to Fungi of Madras—X . . . . . T. S. Ramakrishnan 63

Nitrification in Relation to Cation Absorption by Plants . . . . . S. V. Desai and B. V. Subbiah 73

Studies in the Adsorption of Bacteria. Part I. Adsorption of *B. subtylis* and *B. coli* by *Kaolin* and *Charcoal* . K. Krishnamurti and S. V. Soman 81

Studies on Foliar Sclereids in Dicotyledons. I. Structure and Ontogeny of Sclereids in the Leaf of *Diospyros discolor* Willd. T. Ananda Rao 92

Importance of Manuring in Potato Crop Raised from Cut Pieces . S. L. Kapoor 99

Fertilization Value of Tank Silts. C. S. Venkata Ram and K. V. Srinivasa Pai 109

No. 3—September 1951

Regeneration in an Indian Earthworm, *Perionyx millardi* Stephenson 1915 . . . . . G. E. Gates 115

Studies on the Common Bamboo-Borer, *Dinoderus ocellaris* Steph. Part III. Biology . . . . . N. L. Sitaraman 148

Additions to Fungi of Madras—XI . . . . . T. S. Ramakrishnan 157

Flowering Habits of *Dichanthium annulatum*, Stapf., *Dichanthium caricosum*, A. Camus and *Bothriochloa intermedia* (Br.) A. Camus . . J. G. Oke 165

Pollen and Embryo-Sac of Two Euphorbiaceæ . . . . . I. Banerji 172

PAGE

No. 4—October 1951

The Inflorescence and Flowers of *Dichrostachys cinerea*, W & A . . . . . C. S. Venkatesh 183

Embryological Studies in the Leguminosæ. III. A Contribution to the Embryology of *Pithecolobium saman* Benth. Syn. *Enterolobium saman* Prain . . . . . V. R. Dnyansagar 188

Studies on the Osmotic Properties of the Eggs and Larvæ of a Brackish-water Polychæte, *Marphysa Gravelyi* Southern . . . B. Krishnamoorthi 199

Studies in Sampling Technique. V. Estimation of Borer Incidence in Sugarcane . . . . . K. L. Khanna and K. S. Bandyopadhyay 210

Nature and Nurture in Yeasts . . . . . M. K. Subramaniam 220

No. 5—November 1951

Genetics in Relation to Plant Breeding . . . . . Arne Müntzing 227

Immunological Study in Avian Malaria (*P. gallinaceum*) by the Anaphylactic Reaction . . . . R. Rama Rao, A. S. Ramaswamy and N. N. De 242

On a New Caligid Parasite, *Gloiopotes zeugopteri*, sp. nov., from *Xiphias zeugopteri*, Lawson's Bay, Waltair . . . T. S. Satyanarayana Rao 248

A Photographic Method for Determining Apical Growth in Fungal Hyphæ . . . . . C. S. Venkatram 256

The Effect of a Single Injection of Thyroxin on the Protein and Nucleic Acids of the Mouse Liver and the Protein of the Mouse Carcass . . . . . A. J. Baxi, Kumud D. Samarth and P. R. Venkataraman 258

Excretion of Vitamin C during Germination of Leguminous Seeds . . . . . A. Sreenivasan and S. D. Wandrekar 267

No. 6—December 1951

Studies on Early Development and Regeneration in Some Indian Marine Sponges . . . . . V. R. Sivaramakrishnan 273

Some Burrowing Fishes from Travancore . . . . K. Krishnan Nayar 311

Studies on Foliar Sclereids in Dicotyledons. V. Structure and Development of the Terminal Sclereids in the Leaf of *Memecylon heyneanum* Benth. . . . . . T. Ananda Rao 329

PLACENTATION IN THE SEA-SNAKE, *ENHYDRINA SCHISTOSA* (DAUDIN)*

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CONTENTS

		Page
1.	Introduction	1
2.	Acknowledgments	2
3.	Historical Resume	2
4.	Material and Methods	3
5.	The Reproductive System	4
6.	The Uterine Chambers	7
7.	The Anatomy of the Blastocyst	8
8.	The Histology of the Placenta—	
	(*a*) The Euryplacental Region	12
	(*b*) The Stenoplacental Region	17
9.	Discussion	21
10.	Summary	29
11.	References	30
12.	Explanation of Plates	31

Introduction

Enhydrina schistosa is a sea-snake belonging to the family Hydrophidæ the members of which are predominantly oceanic in habit. As the open sea is not a habitat suited for eggs being laid out to hatch as on dry land, the sea-snakes have perforce to retain the eggs within the body till the young ones complete their development and are ready to be born. "Viviparity," in the broadest sense of the word, seems to be nearly universal in the family. As direct observation of the birth of young sea-snakes is possible only in marine aquaria, the phenomenon has been recorded only very rarely (Raj, 1926). It appears likely that *Laticauda colubrina* and one or two other species of this genus which occur close to the shore have not progressed beyond the oviparous stage (Smedley, 1930, 1931; Smith, 1930), but the great majority of the species included in the family is either ovo-viviparous

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or truly viviparous. There is scope for wide variation among the species in the degree of adaptation in response to the new mode of reproduction, but the phenomenon appears to remain largely unexplored. The subject has been invested with interest from the fact that true allantoic placentation has been described in several species of viviparous reptiles in the past sixty years, and these studies of reptilian placentation throw light on current theories on the mode of origin of the placenta in mammals.

It seemed desirable, therefore, to investigate the adaptive condition in the commonest species of sea-snakes to begin with. Two readily available species at Madras, *e.g.*, *Enhydrina schistosa* (Daudin) and *Hydrophis cyanocinctus* Daudin have been investigated. *E. schistosa* has not only true viviparity but also a distinct type of allantoplacenta, the characteristics of which are quite unlike those recorded by previous workers. The present account deals only with *E. schistosa*, the placenta of the other species being reserved for description in a separate communication.

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HISTORICAL RESUME

Since the publication in 1891 of the classical work of Giacomini on the development of *Chalcides tridactylus* (= *Seps chalcides*) it has been known that a true allantoplacenta occurs in some of the Reptilia, doubtless in the course of the normal evolutionary process independently of the Mammalia. Giacomini described also the placenta of *Chalcides ocellatus* in 1906. Though Giacomini's original descriptions were not available to the present author the handsomely illustrated paper on the placenta of *Chalcides tridactylus* by Cate-Hœdemaker (1933) was helpful in studying the placenta of *E. schistosa*.

Later research in this field has been carried out mostly on species from Australia and New Zealand. The work of Flynn (1933) on *Tiliqua*

scincoides, of Harrison and Weekes (1925) on *Lygosoma entrecasteauxi*, of Weekes (1927–1935) on several species of Lacertilia and two species of Ophidia and of Boyd (1942) on *Hoplodactylus maculatus* may be mentioned here. Weekes's investigations have covered a wide range of species. Her final paper (Weekes, 1935) reviews and discusses the results of ten years' work and provides a comprehensive summary, which should be of very great help to every investigator of reptilian placentation. Two terrestrial snakes, *Denisonia superba* and *D. suta*, also figure in Weekes's list. The present work is, however, the first record of allantoplacentation in the *Hydrophidæ*.

Material and Methods

The material for this study, consisting of 10 embryos of *Enhydrina schistosa*,* was obtained from pregnant females during November and December 1939. A total of 55 specimens was dissected between August 1939 and September 1941 of which 20 were males, 26 were non-pregnant females and 9 were gravid females. The 2 earliest obtained embryos were 194 mm. long and the remaining 8 ranged from 264 mm. to 286 mm. in length. The stages of development reached in the latter are practically identical and form the basis for the description of placentation given below. Bouin's fluid (aqueous) gave very good fixation. Zenker's fluid (with acetic acid) and Formol bichromate mixture were the other fixatives used. Dissections of the uterine chamber and the fœtal membranes were performed under a Zeiss Binocular microscope. Portions of the uterine wall and the allantoic membranes were separated from one another, stained in alum-carmine and mounted whole. It was not found necessary to cut microtome sections of the complete uterine chamber which was quite large in size (35 mm. across). The uterine wall, the chorio-allantoic membrane and the inner allantoic membrane remained adherent to one another in the form of a single sheet which was rolled up and sectioned. Other parts of the fœtal membranes and the yolksac were studied by sectioning suitable pieces. The pre-pregnant and post-pregnant oviducts of the females and the testes of the males were also sectioned to study the seasonal changes in the histological characters. Sections cut varied from 5 to 10 microns in thickness. Heidenhain's Iron Hæmatoxylin followed by Orange G gave very satisfactory results. Delafield's Hæmatoxylin and Eosin, Mann's Methylblue Eosin and Mallory's triple stain were also occasionally used.

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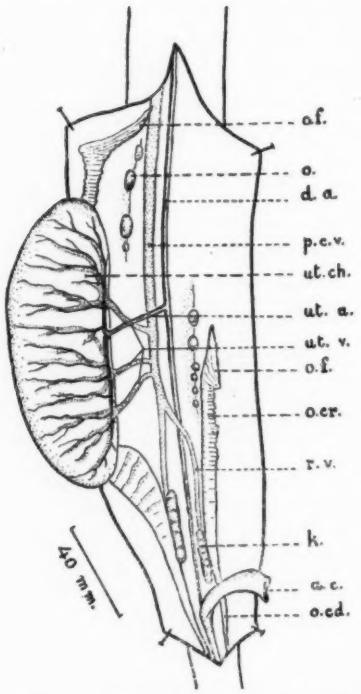
* The genus *Enhydrina* includes only a single species for which three specific names have been proposed at different times. According to Smith (1926, 1943) *E. schistosa* has priority over *E. valakadyn* and *E. bengalensis*, which should be treated as synonyms.

The Reproductive System

The ovaries are elongated bodies 50 to 100 mm. in length. They are ridge-like and hang from the dorsal wall of the body cavity immediately mesial to the oviducts (Text-Fig. 1). The cranial end of the ovary is at the same level as the tip of the oviducal funnel. The right ovary which is a little longer than the left, is placed a little farther forwards. The ovarian eggs are of all sizes, the largest observed being 15 to 20 mm. in length. They are not grouped together at the two ends of the ovary but are scattered irregularly. The full-grown egg about to be shed from the ovary was observed only once. It was 70 to 75 mm. long and extremely soft and flabby. The oviducal funnel on the same side appeared to be very large, the funnel mouth



Text-Fig. 1. *Enhydrina schistosa*, pregnant female with a single uterine chamber.

a. c.	alimentary canal, cloacal region.	*o. f.*	oviducal funnel.
d. a.	dorsal aorta.	*p.c.v.*	postcaval vein.
k.	kidney.	*r. v.*	efferent renal vein.
o.	ovary.	*ut. a.*	uterine artery.
o cd.	oviduct, caudal tubular region.	*ut. ch.*	uterine chamber.
o. cr.	oviduct, cranial tubular region.	*ut. v.*	uterine veins.

being very close to the ripe egg and stretched wide open. Whether active movements of the funnel wall assist in the entry of the egg and its passage into the oviduct is difficult to determine. The unripe egg 20 mm. long and the ripe egg 70 mm. long greatly differed in colour and consistency. The former was quite firm and pale ivory in colour and the latter very flaccid and distinctly yellow in colour. The primary egg-membrane does not increase in thickness with the growth in size of the egg. While it gives a firm outline to the small volume of yolky material in the unripe egg, it is not strong enough to hold firmly the vastly greater amount of yolky material in the ripe egg. The change in colour from ivory to yellow might be due to the difference in composition of the yolk deposited in the later stages or perhaps to the greater percentage of water present.

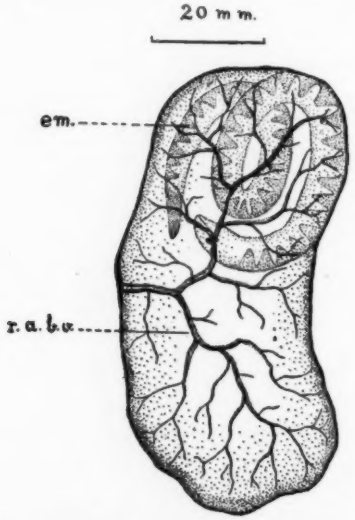
Corpora lutea were observed in the ovaries of all the pregnant snakes and of some of the non-pregnant ones. The yellow luteal bodies were usually large, oval in outline, 10 mm. long and 4 to 6 mm. broad, and had a median longitudinal furrow where the follicle had ruptured and healed.

The oviducts are two tubular bodies placed alongside of the alimentary canal (Text-Fig. 1). The caudal end of each oviduct opens into the cloaca from its dorsal aspect, and the cranial end opens into the body cavity by a large elliptic thin-walled oviducal funnel with its mouth 40 to 50 mm. long directed ventrally. The wall of the funnel is delicate, translucent and much creased. The right oviduct is invariably longer than the left, the difference in length being as much as 40 mm. As the oviduct is pleated over a part of its course, its actual length (apart from its elastic extensibility) is much greater than its apparent length (240 to 280 mm.). The oviduct is suspended from the dorsal wall of the body cavity in a fold of the peritoneum known as the mesometrium. The line along which the mesometrium meets the wall of the oviduct is taken to be the morphological mid-dorsal line of the oviduct. In the mid-ventral line the peritoneum projects as a ridge which is 2 to 20 mm. broad. This—the so-called ventral mesometrium—has no connection with the ventral bodywall. The oviduct hangs freely in the body cavity and is attached on the dorsal side only. The dorsal and ventral mesometria are of great assistance in orientation. As the swollen oviducts in a gravid female are apt to roll to one side or the other in dissection the determination of the morphological dorsal and ventral surfaces is facilitated by referring to the position of the mesometria. This is true also when handling fixed material.

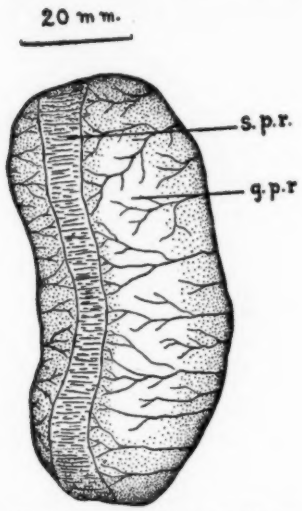
The oviduct reveals three main regions to naked eye examination. Histological study confirms the existence of the three regions which are, (1)

the funnel region which includes not only the thin-walled terminal part but a short length (20–30 mm.) of the tube adjacent to it, (2) *the cranial tubular region* where uterine functions are assumed during pregnancy, and lastly (3) *the caudal tubular region* which seems to be purely efferent in function. In the caudal region the oviduct is placed close to the alimentary canal and the mesometrium is narrow and tough; the walls of the oviduct are thick, with its lumen cylindrical or slightly flattened in section. In the cranial region on the other hand, the mesometrium is broad and elastic and the tubular part is pendulous; the oviducal wall is comparatively less thick, the lumen is very wide and the tube is collapsed into a strap-like shape. Whereas the highly elastic mesometrium runs a straight course, the less elastic oviducal tube of the cranial region runs a sinuous course, being folded like the wall of a concertina.

The reproductive organs exhibit seasonal changes in size. Mature individuals of identical size obtained in different parts of the year showed striking differences in the degree of development of the oviducts or testes. In the mature non-pregnant females obtained during November and December the oviducts have greatly thickened walls plentifully beset with glands, but in the other months they are reduced in size with less thick walls. The males obtained prior to November were very stout and full of fat-bodies, but the testes were small, hardly 10 to 20 mm. long. During



TEXT-FIG. 2. *Enhydrina schistosa*, dorsal view of a blastocyst, showing the coiled embryo (*em.*) and the right allantoic artery and vein (*r.a.b.v.*).



TEXT-FIG. 3. *Enhydrina schistosa,* ventral view of a blastocyst, showing the Euryplacental region (*g.p.r.*) and the band-shaped Stenoplacental region (*s.p.r.*) or chorio-omphalo-allantoic membrane.

November and December the testes were large, up to 100 mm. in length and 20 mm. in diameter. In the later months the testes had shrunk in size and the fat-bodies were greatly depleted. These observations clearly indicate annual cyclical changes and the existence of a definite season for reproductive activity but the duration and the limits of the reproductive season can be determined only on the basis of much larger data.

THE UTERINE CHAMBERS

The embryos when present were invariably contained in the cranial region of the oviduct where uterine characteristics were developed. The funnel region and the caudal region never accommodated embryos. The oviducal wall was stretched to form one or two ovoid swellings at the points where the embryos were lodged (Text-Fig. 1). Giacomini referred to these chambers in *Chalcides tridactylus* as " incubatory chambers." In the present account, the more suitable term " uterine chambers " is used.

The wall of the uterine chamber is transparent in the living condition. The arrangement of the embryo, the yolk-sac, etc., within the chamber is easily seen and the course of the main blood-vessels is followed without difficulty. The uterine blood-vessels and the embryonic blood-vessels are in two distinct layers the latter being below the former. The ovoid chamber, which is about 35 mm. broad and 70 to 80 mm. long, is very soft and yielding.

It is therefore, apt to assume various shapes during fixation which also renders the uterine wall opaque.

The uterine blood-vessels are arranged as follows. Each uterine chamber is supplied by a single large artery springing from the dorsal aorta and travelling down along the mesometrium (Text-Fig. 1). On reaching the chamber the artery divides into two main branches running fore and aft giving off other branches towards the sides which run parallel to one another downward around the uterine chamber (Plate II, Fig. 4) subdividing as they go. A prominent band-shaped network of blood-vessels is noticed in the mid-ventral line along the axis of the uterine chamber, from end to end. It is seen better when the ventral mesometrium is moved aside. In the uterine wall which has been stripped off from the embryonic tissues, the inner surface reveals the vascular band quite conspicuously. This vascular band belongs to the stenoplacental region (*vide infra*). The course of the smaller veins is more or less parallel with that of the arteries. At the dorsal surface of the uterine chamber the bigger veins unite to form 2 or 3 veins which enter either into the efferent renal vein or into the inferior vena cava which arises anteriorly by the junction of the right and left efferent renal veins.

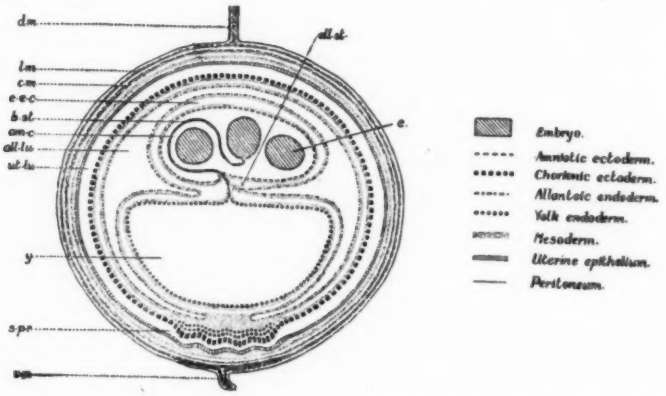
The uterine chamber of *E. schistosa* appears to be a transitory structure. Giacomini (1891) observed incubatory chambers in the oviduct of *Chalcides tridactylus* even three months after the birth of young. Weekes (1927) found the division of the oviduct into chambers persistent in *Lygosoma quoyi*. In *E. schistosa* this persistent division of the oviduct into chambers does not seem to occur. One of the individuals examined could be easily recognised to be a post-pregnant female. From examination of the sections of the oviduct it was obvious that the inner epithelium had undergone very little change from the condition described as placental in the present account. Very little time had elapsed, apparently, after the expulsion of the embryos. But the uterine muscles had contracted strongly removing all external indications of division into chambers, and throwing the internal mucosa into ridges and furrows. In still other oviducts which were recognizably post-pregnant it was observed that the uterine epithelium had lost all traces of placental character. From these observations it appears very probable that the post-partum contraction of the uterus of *E. schistosa* takes place at such a rapid rate that the normal appearance is completely resumed before the next gestation period.

The Anatomy of the Blastocyst

The term " blastocyst " is used here (as in Mossman 1937) to refer to the embryo together with its envelopes, *i.e.*, to all the embryonic tissues

in their entirety contained within one uterine chamber. Besides the embryo and the yolk-sac, the blastocyst comprises certain delicate membranous structures derived from the amnion, the allantois and the chorionic ectoderm, the disposition of which will be indicated below.

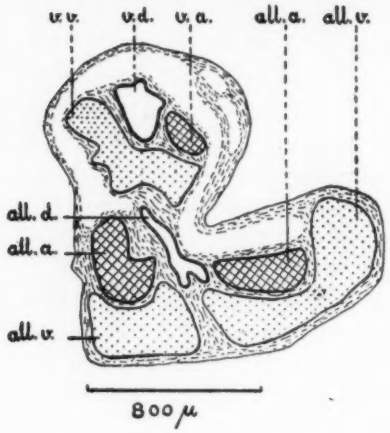
The embryo is a miniature of the adult exhibiting the same beak-like curve of the upper jaw and the same arrangement of alternate bands of dark brown and bright yellow on the dorsal aspect which merges into a uniform white on the ventral aspect. It displays considerable powers of movement within the uterine chamber in the fresh condition and consequently assumes during fixation the position it happens to be in. The yolk-sac is ventral to the embryo and fills up half of the uterine chamber or a little more (Text-Fig. 4). The embryo–yolksac axis generally coincides with the mesometrial vertical axis but may be slightly tilted to one side.



TEXT-FIG. 4. *Enhydrina schistosa*, diagrammatic transverse section through a uterine chamber.

all. lu.	allantoic lumen	*e.*	coiled embryo in section		
all. st.	allantoic stalk	*e. e. c.*	extra-embryonic coelom		
am. c.	amniotic cavity	*l. m.*	longitudinal muscles		
b. st.	body stalk	*s. p. r.*	stenoplacental region		
c. m.	circular muscles	*ut. lu.*	uterine lumen		
d. m.	dorsal mesometrium	*v. m.*	ventral mesometrium	*y.*	yolk

The amnion is a very delicate transparent membrane, closely adherent to the surface of the embryo in the fixed specimens. In the living condition, a viscid fluid fills up the space between the embryo and the amnion. The combined yolk-sac stalk, the vitelline blood-vessels, the allantoic duct and the allantoic blood-vessels, known as the " body-stalk," is about 25 mm. in front of the cloacal aperture of the embryo. The allantoic blood-vessels



TEXT-FIG. 5. *Enhydrina schistosa*, transverse section through the body stalk of the embryo (*c l.* sketch, partly diagrammatic).

all. a.	allantoic artery	*v. a.*	vitelline artery
all. d.	,, duct	*v. d.*	,, duct
all. v.	,, vein	*v. v.*	,, vein

include a pair of arteries and a pair of veins, one of each pair (corresponding to the right side of the embryo) being somewhat larger than the other. The body-stalk is 15 to 18 cm. in length and in a few instances, takes a turn or two around the trunk of the embryo before reaching the yolk-sac. Beyond the yolk-sac, the allantoic duct parts company from the vitelline duct and curves outward towards the periphery where it expands into the allantoic vesicle (Text-Fig. 4).

In the stage of development under reference the allantois is fully formed and extensive, completely investing the embryo and the yolk-sac. In the Lacertilia in general (Hrabowski, 1926) the allantois is confined to the dorsal half of the blastocyst above the yolk-sac but in *E. schistosa* as well as in the two species of *Denisonia* studied by Weekes (1929) the allantois grows down along the interstice between the yolk-sac and the chorionic ectoderm so as to completely invest the yolk-sac.

The allantoic vesicle has proximal and distal walls. The proximal wall known as the *inner allantoic membrane*, remains partly unattached, partly adherent to the amniotic membrane in the upper regions of the blastocyst and united with the wall of the yolk-sac in the lower regions. The distal wall is fused over its entire extent with the chorionic ectoderm which is the outermost covering of the blastocyst, to form the compound structure, the

chorio-allantoic membrane. A limited space between the outer and inner allantoic membranes is actually present in the upper half of the blastocyst but is potential in the lower half where the two membranes are in contact.

The extension of the allantois into the ventral half of the blastocyst needs a few words of explanation. At the cranial and caudal ends of the uterine chamber the edges of the allantois appear to terminate abruptly at the equatorial line. On the two sides however they appear to continue down to the ventral pole without meeting, leaving between them a long band-like area stretching ventrally from end to end of the blastocyst. This area corresponds to the stenoplacental region (*vide infra*). The allantoic endoderm stops short on either side but the allantoic mesoderm is continuous from one side to the other and indeed forms a specially thick strand traversed by the blood-vessels of the stenoplacental region (Text-Fig. 8 and Plate II, Fig. 5).

Of the two pairs of allantoic blood-vessels mentioned earlier the smaller pair travels downward along the inner allantoic membrane to supply the stenoplacental region. The larger right pair crosses the allantoic cavity directly reaching the chorio-allantoic membrane along which it goes upwards to branch and supply the entire upper half of the blastocyst and most of the lower half of the farther side (Text-Fig. 2).

There is no trace of the shell-membrane between the blastocyst and the uterine wall. In some of the uterine chambers what appear to be the remains of the shell-membrane are present in the form of two circular patches placed at the two ends of the ovoid chamber where the wall of the chamber is continued into the unmodified part of the oviduct. It is to be presumed that the shell-membrane has been completely absorbed in the course of development.

Placental modification is found over the entire extent of two contiguous surfaces, *viz.*, the inner surface of the uterine chamber on the one hand and the outer surface of the blastocyst on the other. Two regions of marked contrast can be recognised in the placenta. The region covering a limited area will be termed the Stenoplacental region* on account of its narrow shape, while the other region including a comparatively much greater area will be termed the Euryplacental region.* These two terms are newly instituted as they are found to be necessary for brief and unambiguous reference to the respective regions.

The Stenoplacental region is a band-shaped area, mid-ventral in position, 3 to 4 mm. broad and as long as the uterine chamber (70 to 80 mm.).

* From Greek *eurus* = broad and *stenos* = narrow.

The maternal and fœtal tissues are not attached together over this area. When they are separated, the inner surface of the uterine wall is seen to be highly vascularised and to have a longitudinally creased appearance. The corresponding area of the blastocyst is but little folded or creased. It has a curiously whitish opaque appearance with sharp outline (Text-Fig. 3, *s.p.r.*) owing to the presence of a layer of vacuolated yolk-endoderm just below the chorionic ectoderm, as will be clear from the histological account given below. The yolk-endoderm also obscures the presence of a rich network of capillaries beneath. The Euryplacental region includes the greater part of the placental area, excluding only the fraction covered by the other region. The uterine wall and the allanto-chorion are firmly attached together over this region and no one who dissects the uterine chamber in the living condition will fail to notice the firm adhesion. The areas in contact are uniformly smooth and without foldings. The vascularisation is not so conspicuous as in the other region.

Histology of the Placenta

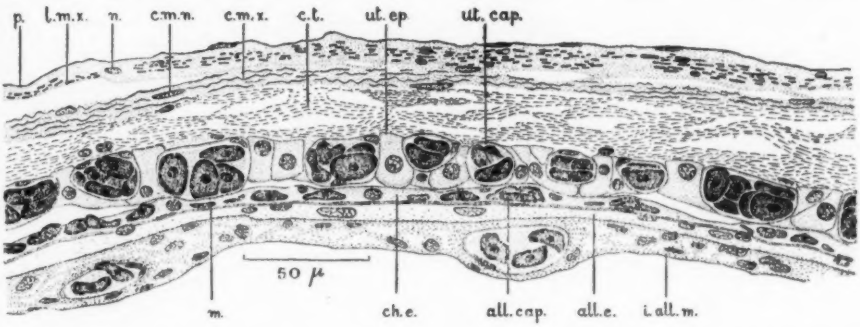
(*a*) *The Euryplacental Region*

The placenta in this region consists of two highly vascularised epithelial sheets which are placed in intimate contact and mutual attachment. The epithelia belong to the uterine wall on the one hand and the allanto-chorion on the other (Text-Figs. 6 and 7).

Maternal part of the placenta.—The uterine wall is composed of the peritoneum externally, the longitudinal and circular muscle layers, the internal layer of connective tissue and the uterine epithelium. In Text-Figs. 6 and 7, the plane of section passes across the axis of the uterine chamber and consequently the circular muscles are cut parallel to the fibres while the longitudinal muscles are cut across. In sectional view, the uterine wall is not uniformly thick all around the uterine chamber (40 to 100 microns) mainly in consequence of the fact that the longitudinal muscles form an interrupted layer composed of large and small bundles. Occasionally, when the plane of section coincides with the course of one of the arteries or veins passing down the sides of the uterine chamber, sections appear nearly 350 microns in breadth which gives a very misleading and exaggerated idea of the thickness of the uterine wall.

The peritoneum is extremely tenuous. Under low magnification (× 80) it cannot be distinguished apart from the longitudinal muscles but under high magnification (× 1,000) it is distinguishable as a thin streak in which the nuclei are few and far apart. In the muscle layers, the fibres are unstriated, the nuclei are oval or sausage-shaped and rounded at the ends.

The outlines of the individual muscle fibres are hardly distinguishable in cross-sections although very thin connective tissue partitions are present which appear to separate groups of muscle fibres. Certain curious sinuous



TEXT-FIG. 6. *Enhydrina schistosa*, transverse section through the uterine wall, allantochorion and the inner allantoic membrane in the eutyplacental region (camera lucida sketch).

all. cap.	allantoic capillary vessel	*l. m. x.*	longitudinal muscle ; streak-like object
all. e.	,, endoderm	*m.*	mesodermal layer
c. m. n.	circular muscle ; nucleus of a muscle fibre	*n.*	longitudinal muscle ; nucleus of a muscle fibre
c. m. x.	circular muscle ; sinuous streak	*p.*	peritoneum
c. t.	connective tissue	*ut. cap.*	uterine capillary vessel
ch. e.	chorionic ectoderm	*ut. ep.*	uterine epithelium
i. all. m.	inner allantoic membrane		

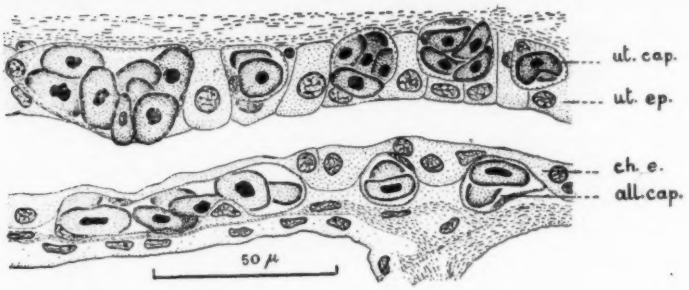
streaks are noticed along the fibres of the circular muscles (Text-Fig. 6, *c.m.x.*) and appear in cross-section in the longitudinal muscles (Text-Fig. 6, *l.m.x.*). In the latter case, while appearing flattened where the muscles are stretched as for example over the sides of the uterine chamber, they are polygonal in the muscles which are not compressed (*e.g.*, the ventral mesometrium). It is difficult to say what these are.* The layer of circular muscles is uniformly thick. The connective tissue layer is variable in thickness, with the nuclei scattered in a matrix of wavy or fibrillar appearance. The uterine epithelium dips down into the connective tissue layer in the form of finger-like ingrowths which are plainly visible in the surface view of whole mounts of the uterine wall as well as in microtome sections. These ingrowths are composed of regular rectangular cells bounding a narrow central space. The cells are not ciliated and do not show glandular activity. They are

* They may be the "strong" and tangential threads described by Heidenhain (Cajal, 1933, p. 247) or the interfibrillar connective tissue fibres (figured in Cajal, 1933, p. 251); the streaks greatly resemble the objects seen (besides the nuclei) in figures 352 and 353 given by Meigs in Cowdry (1928). Though Meigs has given the figures, he has made no particular reference to the objects or offered any remarks on them.

referred to as " glands " and it is probable that they functioned as simple tubular glands in the pre-pregnant uterus.

The uterine epithelium is composed of a single layer of cells which are cuboidal or rectangular in shape and 15 to 20 microns broad in sections. Cell-limits are clear under high magnification (× 1,200) and the cell-nuclei are prominent. The nuclei possess a moderate amount of chromatin and one or two nucleoli. The cell-margins bear no cilia and no type of border differentiation of the cells (rod-border, brush-border, " schlusleiston ") is observable. A network of capillaries is embedded within the thickness of the epithelium, and in consequence the epthelial cells are pushed apart or otherwise disarranged. The erythrocytes within the capillaries are darkly stained by hæmatoxylin but the uterine epithelial cells are not stained except for their nuclei. As a result, the capillaries stand out conspicuously like beads in a rosary (Text-Fig. 6), even under low magnification (× 80). The individual capillary is circular or oval in cross-section. The endothelial wall is very delicate and possesses flattened nuclei. The blood corpuscles are tightly packed in the lumen of the capillaries and are therefore often distorted in form. The free red blood corpuscle is biconvex, oval in outline and nucleated. The capillaries are not uniformly developed all over the extensive area of the euryplacental region. At a few points, the capillaries lie at the base of the epithelial cells and hardly at all enter into them; but this condition is very far from general. Over the greater part of the area, the capillaries extend into the epithelium, taking up a position between the cells by pushing them apart, till only very attenuated extensions of the cells remain intervening between the capillary endothelia and the uterine lumen (Text-Fig. 6). Where the capillaries are large and numerous (Text-Fig. 7) they bulge a little into the uterine lumen. These capillaries are truly " exposed " to the uterine lumen; rather, their endothelial walls are in direct contact with the allanto-chorion of the embryo, and there is no trace of intervening uterine epithelium between them. At the points where the arterioles connect with the capillary net (Plate I, Fig. 1) the capillaries form the largest and closest network with the uterine epithelium least in evidence. Conversely, where the capillary network is sparse, the uterine epithelial cells are most clearly in evidence.

Fœtal part of the placenta.—The chorio-allantoic membrane is 12 to 20 microns thick. It includes the chorionic ectodermal epithelium, an intermediate mesodermal layer and the allantoic endodermal epithelium. Text-Fig. 6 represents an instance in which the inner allantoic membrane remains adherent to the chorio-allantoic membrane. The cells of the chorionic



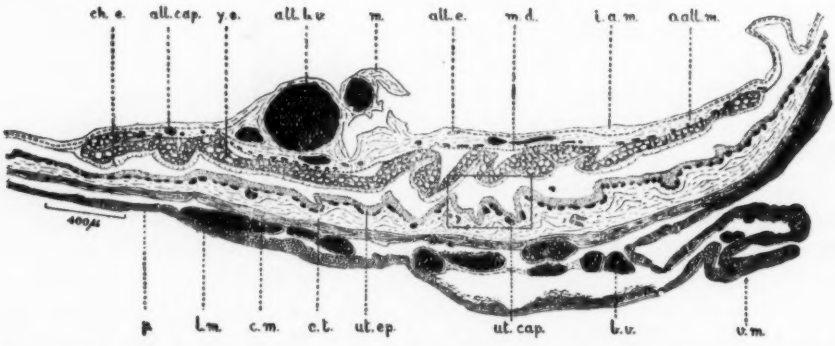
TEXT-FIG. 7. *Enhydrina schistosa*, transverse section through the uterine wall and the allanto-chorion in the euryplacental region to show an exposed uterine capillary and a large allantoic capillary (camera lucida sketch).

all. cap. allantoic capillary *ut. cap.* uterine capillary
ch. e. chorionic ectoderm *ut. ep.* uterine epithelium

ectoderm are spindle shaped in sections on account of their being greatly flattened. The individual cell is 3 to 5 microns thick near the centre where the nucleus is contained and very much thinner towards the edges. As the nuclei are on an average 30 microns apart, this distance may be taken to be the approximate diameter of the chorionic ectoderm cells as seen in surface view. Cell-limits can be seen only very rarely. A network of allantoic capillaries lies immediately below the chorionic ectoderm, the individual capillary having a much flattened oval outline in sections. The capillaries are all conspicuous because of the dark stain taken by the erythrocytes. The delicate endothelial walls and their flattened nuclei can be recognized with some difficulty. Over some of the capillaries there is no extension of the chorionic ectodermal cells and so, there is true "exposure" of the allantoic capillary. Such a condition is observed here and there but not uniformly all over the euryplacental region. Nor do the exposed allantoic capillaries correspond in position with the exposed uterine capillaries. The chorionic ectoderm and the allantoic capillaries together form a layer of uniform thickness (5 microns), below which is a thin layer (1 or 2 microns) of connective tissue. Though this tissue represents both the chorionic mesoderm (parietal wall of the extra-embryonic cœlom) and the allantoic mesoderm, no visible distinction exists. Underlying this mesoderm is the allantoic endoderm and next to it is the inner allantoic membrane. The endodermal layer is 5 to 8 microns broad with large, elongated, irregularly scattered nuclei; cell-limits are indistinguishable. This layer represents the fused endodermal epithelia of the two allantoic membranes which are in contact. The inner allantoic membrane is composed of a very loose spongy layer of

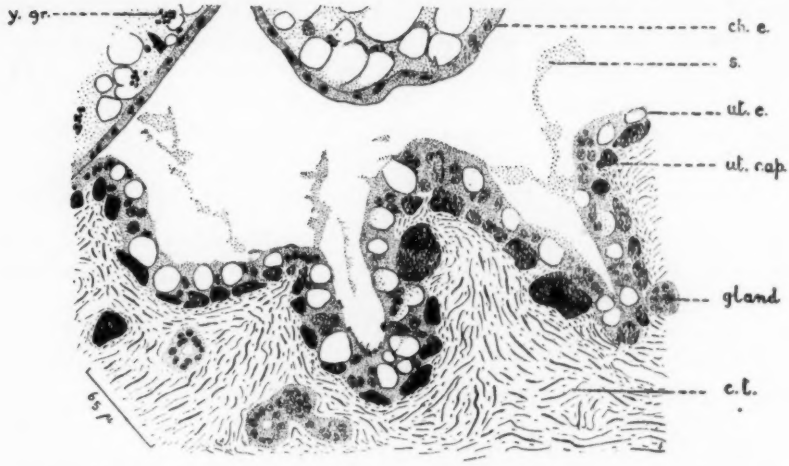
connective tissue, 10 to 50 microns thick. The smaller arteries and veins which supply the allantoic capillary network, take their course along the chorio-allantoic membrane which bulges inwards at these points. Blood-vessels are present on the inner allantoic membrane also, but they are fewer.

The attachment of the chorionic ectoderm to the uterine epithelum is abundantly clear in dissection and in sections. Plate I, Fig. 3 shows the ectoderm cells in the form of conical projections as a result of the separation of the allanto-chorion from the uterine wall by mild force. The ecto-derm cells are clearly in strong adhesion to the uterine epithelial cells. But even the most careful examination failed to reveal the pseudopodial proces-ses of fœtal cells introduced into the maternal cells as shown by Hill (1900) in *Dasyurus viverrinus*, by Flynn (1923, p. 76) in *Tiliqua scincoides*, and by Weekes (1927, p. 532) in *Lygosoma quoyi*. Even after sectioning, the allanto-chorion remained in close contact with the uterine wall as in Text-Fig. 6, but occasionally the two membranes move apart (see Text-Fig. 7).



TEXT-FIG. 8. *Enhydrina schistosa*, transverse section through the entire width of the stenoplacental region, showing the uterine wall and the chorio-omphalo-allantoic membrane (camera lucida sketch).

all. b. v.	allantoic blood vessel	*m.*	mesodermal tissue
all. cap.	,, capillary	*m. d.*	mesoderm, dense layer
all. e.	,, endoderm		(indicated by a broken line)
b. v.	uterine blood vessels	*o. a. m.*	outer allantoic membrane
c. m.	circular muscles	*p.*	peritoneum
c. t.	connective tissue layer	*ut. cap.*	uterine capillaries
ch. e.	chorionic ectoderm	*ut. ep.*	uterine epithelium
i. a. m.	inner allantoic membrane	*v. m.*	ventral mesometrium
l. m.	longitudinal muscles	*y. e.*	yolk-endoderm



TEXT-FIG. 9. *Enhydrina schistosa.* The area marked in Fig. 8 under greater magnification to show the glandular cells and their secretion (camera lucida sketch).

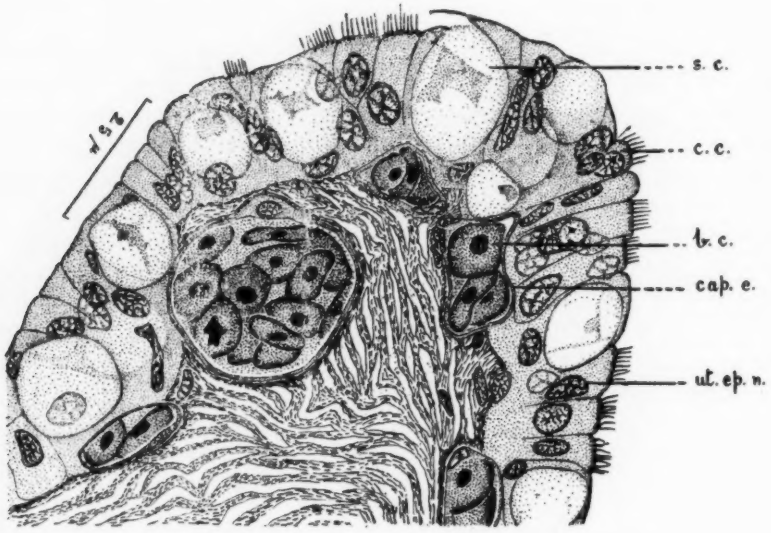
c. t. connective tissue layer
ch. e. chorionic ectoderm
g. "gland"
s. secreted material
ut. cap. uterine capillary
up. ep. uterine epithelium
y. gr. yolk granules

(*b*) *The Stenoplacental Region*

The position of this region and its naked-eye appearance have been referred to already. It is a band-shaped area, situated on the ventral aspect of the uterine chamber, 3 to 4 mm. broad and as long as the uterine chamber (70 to 80 mm.). The maternal and fœtal tissues are in close contact but unattached. The area is folded to a certain extent along the length of the blastocyst with the convexities of the fœtal tissues fitting into the hollows in the folds of the uterine wall.

Maternal part of the placenta.—The uterine wall includes as usual the peritoneum, the muscle layers, the connective tissue layer and the uterine epithelium. In Text-Fig. 8 the plane of section passes at right angles to the axis of the uterine chamber and the full width of the stenoplacental region is included. The uterine wall is nearly 170 microns across in section which indicates a greater thickness of the uterine wall in this region in comparison with the euryplacental region. The longitudinal muscles are in thicker bundles especially on the ventral mesometrium; the blood-vessels are more numerous; the circular muscles form a slightly broader band; the connective tissue layer is not only broader but is raised inwards to form ridges and furrows covered by the uterine epithelium rendering the

area of the uterine epithelium larger than it would have been if the surface were smooth. Gland-like pockets similar to those found in the euryplacental region are found here also (Text-Fig. 9).



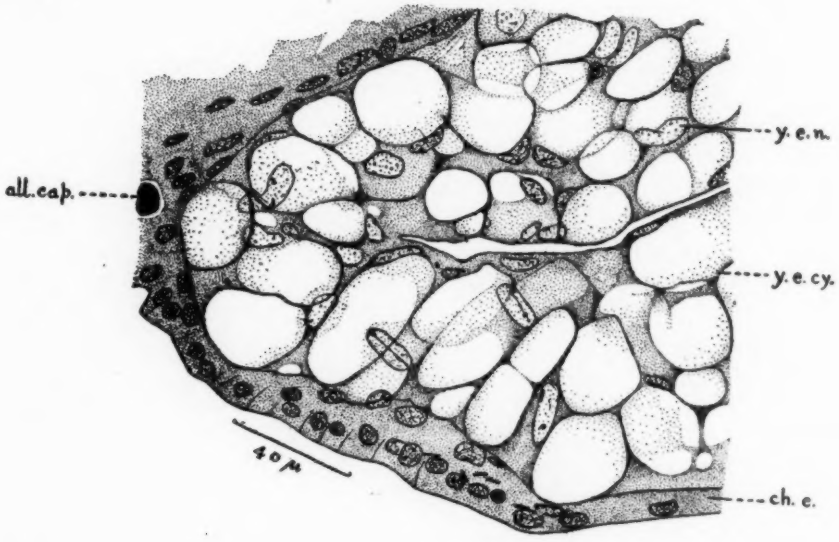
TEXT-FIG. 10. *Enhydrina schistosa*, uterine epithelium of the stenoplacental region (camera lucida sketch).

b. c. blood corpuscle — *c. c.* ciliated cell
cap. e. capillary endothelium — *s. c.* secretory cell
ut. ep. n. uterine epithelial nucleus

The uterine epithelium is a single layer of cells, 20 to 25 microns thick. It includes cylindrical, columnar, ciliated cells and broad cells which possess, in most cases, a single large secretory vacuole (Text-Fig. 10). The secretory material is often found within the cell-vacuole, and material of an identical appearance, lies free in the lumen in the form of a streak just above the uterine epithelium (Text-Fig. 9); direct continuity between the two can be seen in some of the sections. In some of the glandular cells, two or three large vacuoles can be seen in the act of merging together into one, but not a single instance occurs of a cell with a large number of small vacuoles filled with secretory substance—a condition which is known to occur as one of the phases in the secretory cycle of cells. Some of the glandular cells have cilia at their free edge which seem to be cast off when the cell discharges its contents. This observation also suggests that ciliated cells and secretory cells are not two

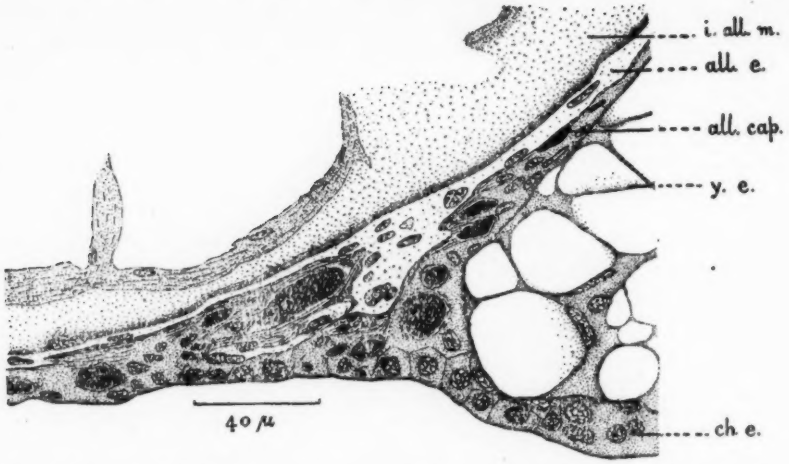
sharply differentiated categories, and that the former are relatively unmodified cells which assume secretory functions at the appropriate time. The secretory cells probably go through one or more cycles of secretory activity, cease to function and undergo cytolysis, as may be inferred from the shreds of cells observed in the uterine lumen. The detachment of complete cells and their disintegration as described by Cate-Hœdemaker (1933) in *Chalcides tridactylus* have not been observed in *E. schistosa.*

Some of the columnar cells appear to lack cilia, which might have been washed away. In the majority of the columnar cells, true cilia are present and under high magnification (× 1,200) their basal granules can be clearly distinguished. For the most part, the ciliated cells and the glandular cells are unevenly mingled together and distributed along the uterine surface. The network of darkly stained capillaries is observed at the base of the uterine epithelium. The capillaries are usually larger in this region than in the eurycplacental region. None of the capillaries penetrates into the epithelium.



TEXT-FIG. 11. *Enhydrina schistosa,* one edge of the chorio-omphalo-allantoic membrane of the stenoplacental region, to show especially the yolk-endodermal layer (camera lucida sketch).

all. cap. allantoic capillary
ch. e. chorionic ectoderm
y. e. cy. yolk endodermal cytoplasm
y. e. n. yolk endodermal nucleus



TEXT-FIG. 12. *Enhydrina schistosa*, transverse section through the marginal transitional zone between the euryplacental region and the stenoplacental region (camera lucida sketch).

all. cap. allantoic capillary
ch. e. chorionic ectoderm
y. e. yolk endoderm
all. e. allantoic endoderm
i. all. m. inner allantoic membrane

Fœtal part of the placenta.—The fœtal tissues of the stenoplacental region appear to be markedly different from the corresponding tissues of the euryplacental region and are not so readily identified as in the latter. Yet, by following any particular layer from the stenoplacental region across the border zone (Text-Fig. 12) into the euryplacental region, its correct identification becomes possible. The chief point of difference lies in the presence of a thick spongy layer of yolk-endoderm adjacent to the chorionic ectoderm layer and intervening between it and the allantoic capillaries. In consequence the chorio-allantoic membrane of the euryplacental region is continuous with the chorio-omphalo-allantoic membrane of the stenoplacental region. The band-shaped sharply bounded whitish area on the ventral aspect of the blastocyst (Text-Fig. 3, *s.p.r.*) corresponds precisely with the chorio-omphalo-allantoic membrane.

The chorionic ectodermal layer is composed of irregularly arranged polygonal cells without stratification into layers. It is variable in thickness (4 to 25 microns) and about 10 microns broad. The cells at the very edge, in sectional view, are more nearly rectangular and regular in arrangement and without cilia. Cell-boundaries are generally clear with the cell-nuclei large, centrally placed and deeply stained. Certain deeply stained (in hæmatoxylin) rod-shaped bodies which are present in the cells of the

chorionic ectoderm (Text-Fig. 11) are absent in the uterine lumen or in the uterine secretion. These bodies may probably be compared to the " uterine stäbchen " of Bonnet or the " hippomanes " found in ungulate placenta (Marshall, 1922, p. 437, fig. 121).

The yolk-sac endoderm is a highly vacuolated layer 70 microns broad in sections. Cell-limits being absent, the cytoplasm is transformed into strands and sheets of complex form studded irregularly with flattened nuclei often of bizarre shapes (Text-Fig. 11). Deeply stained globular bodies of various sizes which are the remnants of yolk-granules, occur singly and in groups. In sections of the yolk-sac there are numerous large granules whereas in the chorio-omphalo-allantoic membrane these are fewer, the yolk-endoderm having been mostly absorbed leaving only scattered remnants.

Adjacent to the yolk-endoderm is the layer of allantoic capillary blood-vessels which are less darkly stained here than elsewhere. They are quite as numerous as in the euryplacental region. Beyond the capillaries is a mesodermal layer, 70 to 80 microns thick, along which run the main blood-vessels of the stenoplacental region. The mesoderm is derived from the allantois and most of it is of a loose texture. Some of it, however, forms a thin dense layer (Text-Fig. 8, *m.d.*) next to the yolk-endoderm and the capillaries. The allantoic endoderm also appears in sections as a thin dense streak (Text-Fig. 8 and Plate II, Fig. 6) which stops short on either side without meeting in the midventral line.

Discussion

The term " placenta " originally applied to the mammalian allantoic placenta, was later extended to associations between the yolk-sac and the uterus in the marsupials, reptiles and elasmobranchs giving rise to the term " Omphaloidean placenta " and even to such atypical instances as the anal nutritive processes of fishes and the branchial attachment of *Salpa* (Mossman, 1937, p. 155). The many definitions of the term " placenta " have been reviewed and discussed by Mossman (1937), but Flynn's definition which applies " to all organs consisting of an intimate apposition or fusion of the fœtal membranes with the uterine wall for the purpose of carrying out physiological processes destined for the well-being of the embryo " seems most suited to the structure representing the placenta in *E. schistosa*.

To Weekes's (1935) three types of placenta must be added a fourth which is present in *Enhydrina schistosa* and *Hydrophis cyanocinctus*.* The main

* Although the placenta of *H. cyanocinctus* is being described in a separate communication it may be mentioned here that the placental condition agrees with that of *E. schistosa* in all the fundamental characteristics including the possession of two distinct placental regions.

Table comparing the type

Type	Examples	Characteristics of the placenta—the Main Area	
		Maternal tissues	Fœtal tissues
I—A	*Lygosoma quoyi* *L. quadridigitatum* *Egernia cunninghami* *E. whitei* *E. striolata* *Mabuja maltifasciatum* *Tiliqua scincoides* *T. nigrolutea* *Chalcides ocellatus* *Hoplodactylus maculatus*	The uterine epithelium is said to be " degenerate "; it is invaded by the enlarged uterine capillaries which are partly exposed, partly covered by a thin layer of cytoplasm. Epithelium not ciliated	The chorionic ectoderm is composed of thin much flattened cells, underlain by numerous enlarged allantoic capillaries. The allantois extends downwards as the yolk-sac decreases in size
		There is attachment between the two epithelia (except in Hoplodactylus). Certain special cells of the chorionic ectoderm serve in attachment.	
I—B	*Denisonia superba* *D. suta*	Do	Do The allantois early extends down to embrace the yolk-sac
II	*Lygosoma pretiosum* *L. ocellatum* *L. metallicum*	Ridges present, small but characteristic, each of one large maternal capillary only. The grooves between the folds are lined by glandular epithelium, non-ciliated	Chorionic ectoderm not reduced, thickened, glandular, uneven, so as to fit into the ridged uterine wall. Allantois occupies only the dorsal half of blastocyst
		Attachment as in Type I	
III—A	*Lygosoma weekesa*	As in type I	As in type I
B	*L. entrecasteauxi*	Do	Do
C	*Chalcides tridactylus*	Do	Do
		The Euryplacenta	
IV	*Enhydrina schistosa* *Hydrophis cyanocinctus*	Nearly same as in Type I but the epithelium does not show signs of " degeneration " ; the cell boundaries are generally clear. Nonciliated	The allantois extends down to embrace the yolk-sac also. Otherwise as in Type I
		There is a firm attachment between the two epithelia	

of Reptilian Placentae

Characteristics of the placenta—the Restricted Area

Degree of folding	Maternal tissues	Fœtal tissues
	Absent	
Folding present	"Over a small area, dorsolateral in position at the posterior end of the blastocyst, the internal face of the maternal wall is raised up into vascular villous ridges round which the fœtal tissue is folded." In every other way this area is similar to the smooth region in its characteristics (Weekes, 1929), p. 42.	
	Absent	
	The elliptic placenta (Mid-dorsal) Apposition only—No attachment	
Folding of maternal wall only	Maternal area composed of complex folds filled with capillaries and lined by a single layer of cuboid enlarged glandular epithelial cells ciliated.	Fœtal area of "enormously" enlarged columnar ectoderm cells with their free surfaces mammillate, non-ciliated, not folded. Capillaries only at the base of the epithelial cells.
Do	As above, folds more complex	As above, chorionic cells ciliated
Do	As above, folds still more complex	Fœtal tissue as above, but folded to fit into grooves between uterine ridges
	The Stenoplacenta (Mid-ventral) Apposition only—no attachment	
Folding present in Enhydrina; absent in Hydrophis	There are ridges and grooves of a simple nature lined by enlarged cells with large secretory vacuoles mixed with which are ciliated non-glandular cells. The capillaries are numerous, but they lie at the base of the epithelium	The chorionic ectoderm is about 10 microns thick and there is below it a layer of modified yolk-endoderm of 70 microns thickness. Below this double sheet are numerous allantoic capillaries. Ridges and grooves correspond to those of uterine wall

features of these four types of placenta, succinctly presented in the accompanying table, would seem to represent different levels of complexity without implying any phylogenetic relationships. While on the one hand there are unrelated genera like *Chalcides* and *Lygosoma* possessing the same type of placenta (*i.e.*, type III) there are species of *Lygosoma* on the other hand which are either with one or the other of the first three types of placenta or are oviparous and aplacental. It seems obvious that the placental condition gives no indication of phylogenetic affinity and that the development of placentation may frequently occur on parallel lines amongst the reptiles. There seems little doubt from the existing evidence on hand that the placenta in the sea-snakes has had an independent origin and evolution with a few hitherto unobserved novel characteristics.

Weekes (1935) has remarked that the placenta of type I is the simplest and the least specialised, but the simplicity seems to be secondary and adaptive. The placenta of type II should perhaps be considered less specialised than types I and III, because it combines in a remarkable degree the salient features of both these two types, namely the superficial position of the capillary characterising type I and the marked development of the uterine and chorionic epithelia of the restricted area which characterises type III. Whereas type I seems to have been derived from type II by the loss of the secretory function of the uterine epithelium and the attenuation of the chorionic epithelium, type III would appear to have been derived by the restriction to a limited area of the high development of the epithelia, and the confinement of the capillaries to the base of the epithelial cells in that area.

The Euryplacental region.—The euryplacental region of *E. schistosa* is fundamentally the same as the placenta of type I which is widely present among placental reptiles. It occurs, for example, in types III and IV also which differ from type I in the presence of an *additional* region with special characteristics. As the placenta of type I consists of a very close approximation of maternal and fœtal blood-streams through the progressive reduction in functional importance of the uterine and chorionic epithelia, its wide occurrence is not surprising. One of the prime needs of the organism developing within the egg, is facility for gaseous exchange for respiration. Such respiratory exchange with the surrounding medium goes on freely in oviparous eggs but the exchange is impeded when the eggs are retained in the oviducts of the mother. Hence, one of the first adaptations to ovo-viviparity is the increase of the vascularity of the uterine wall around the contained egg, so that the exchange of respiratory gases might go on—albeit through the shell-membrane—between the embryo and the maternal blood (Jacobi, 1936). When this adaptation proceeds further, the shell-membrane gradually

disappears and an increasingly closer approximation of the maternal and fœtal circulations is attained, leading ultimately to the placenta of type I. Both Giacomini (1891) and Weekes (1935) have attributed a respiratory function to this type of placenta, which is discharged by the euryplacental region in *E. schistosa* also. Nevertheless it is open to question whether respiration is the *sole* function of the region. It seems possible that diffusion of a certain amount of nutritive material from the maternal blood-stream to the fœtal might take place through the intervening endothelial walls, such diffusion constituting " Hæmotrophic nutrition " on a small scale (*vide infra*).

Some of the points in which the euryplacenta of *E. schistosa* differs from that of other forms may now be reviewed. Cate-Hœdemaker (1933) states that in the " paraplacental region " (corresponding to the euryplacenta) of *Chalcides*, both the uterine epithelium and the chorionic epithelium are syncytial. Weekes (1927, p. 532) mentions that in all the reptiles with a placenta of type I, there is partial degeneration of the two epithelia. The uterine epithelium of *E. schistosa* is neither syncytial nor degenerate. Broken down nuclei are not seen and cell-limits are observable. Nor does it appear that the degenerate syncytial condition is reached in the final stages of the gestation period, because the post-pregnant uterus (mentioned earlier as from a female which had given birth to young very shortly before capture) when sectioned and examined revealed clearly a condition of the uterine epithelium in no way different from the euryplacental region. Cate-Hœdemaker (1933) mentions the presence of a stäbchensaum along the free border of the epithelia. The stäbchensaum is probably identical with the " rod-border " among the several types of cell-borders which have been listed by Newell and Baxter (1937). Among vertebrates, the differentiation of a cell-border has been described for the epithelium of the small intestines only; its occurrence in the reproductive tract of *Chalcides tridactylus* according to Cate-Hœdemaker (1933) is therefore noteworthy. In *E. schistosa*, the occurrence of cilia or of border differentiation is not observed, in the eury-placental region. Cate-Hœdemaker (1933) uses the term "endothelio-endothelial placenta " for this region, thus extending the method of classifying placental types originated by Professor Grosser (De Lang, 1933). This term is not adopted here, because the exposure of the capillaries occurs here and there and not uniformly all over the euryplacental region.

The Stenoplacental region.—The placenta of type III is found in three species of Lacertilia and *E. schistosa* (and *H. cyanocinctus*) resemble them in possessing a restricted area with special characteristics in addition to the type I

placenta over the main area. But the stenoplacental region of the two sea-snakes differs so markedly from the " dorsal elliptic placenta " of type III that it must be ranked as a new type, type IV. The position of the restricted area is diametrically opposite in the two cases, being mid-dorsal in type III and mid-ventral in type IV. The degree of development of the epithelia is in strong contrast. Whereas in type III the chorionic ectoderm is a single layer of little modified or folded elongated cells (Weekes, 1935, Plate V), in type IV the chorion is composed of more than one layer of cells and is noteworthy for its close association with a layer of vacuolated syncytial yolk-endoderm, the two layers together being folded to a certain extent (Text-Fig. 8). The uterine epithelium in type III is greatly increased in area to form complex projecting folds filled with capillaries (Weekes, 1935, Plate V), whereas in type IV it is folded to a lesser extent and is remarkable, chiefly, for the presence of the glandular cells.

The glandular cells of the uterine epithelium are rather like goblet cells. Cate-Hœdemaker (1933) does not report the occurrence of glandular cells in the uterine epithelium of *Chalcides tridactylus*. Harrison and Weekes (1925, p. 477) found in *Lygosoma entrecasteauxi* cells having densely granular dark-staining cytoplasm without vacuolation or glandular activity and Weekes found granular and non-vacuolated uterine epithelial cells in *Lygosoma weekesæ* (Weekes, 1929, p. 55). Glandular activity seems to be in much clearer evidence in sea-snakes than in the lizards cited above.

The presence or absence of ciliated epithelium in *E. schistosa* and the other species is shown in the accompanying table.

Species	Uterine epithelium	Chorionic epithelium
Lygosoma entrecasteauxi	Cilia present	Cilia present
Lygosoma weekesæ and *Enhydrina schistosa*	Cilia present	Cilia absent
Chalcides tridactylus	Cilia absent but stäbchensaum present	Cilia absent but stäbchensaum present

The suggestion made earlier (p. 19) that the glandular cells after a period of activity might undergo cytolysis and be absorbed by the fœtal tissues, is in harmony with the observations of Cate-Hœdemaker (1933, p. 328*). The presence of vacuoles in the chorionic-ectoderm has not been observed in the Australian Lacertilia or in *E. schistosa* but it is clearly shown in Fig. 31

of Hœdemaker's paper (1933).* In the same figure a high degree of degeneration of the maternal tissues is shown and this is said to facilitate the separation of the fœtal from the maternal tissues immediately prior to birth. Such degenerative changes appear to be absent in *E. schistosa* as the young are born by rupture of the fœtal membranes which remain unexpelled in the uterus. For, the examination of the post-pregnant uterus mentioned earlier revealed the presence of loose fragments of fœtal membranes within it, one of the pieces being clearly recognised as a portion of the chorio-omphalo-allantoic membrane. The fœtal membranes are ejected by *Chalcides tridactylus* as " after-birth " and are consumed by the newly born young (Cate-Hœdemaker, 1933, p. 318).

The function of the stenoplacental region is clearly nutritive. The rich vascularisation of the uterine wall and the fœtal wall in this region indicates a high degree of metabolic activity which is obviously of a secretory nature on the maternal side and of an absorptive nature on the fœtal side. The material secreted by the uterine epithelium does not appear to be mere water, for, a viscous residue is visible even in sections. As the nutritive activity is carried out by two contiguous cellular layers it should be termed " histiotrophic nutrition ", in contrast to " hæmotrophic nutrition " where nutritive materials pass directly from the maternal blood to the fœtal by diffusion through barriers of extreme tenuity. Both types of nutrition are known to occur in mammalian placentae, the former being found in epithelio-chorial and the latter in the hæmo-chorial types.

The yolk-sac placenta occurs invariably in all the viviparous reptiles. In the Lacertilia, the omphalo-placenta and the allanto-placenta coexist for a considerable period, the latter being confined to the upper half of the blastocyst. In the Ophidia which have been investigated, the duration of the yolk-sac placenta is cut short by the growth of the allantois downwards and its interposition between the yolk-sac epithelium and the chorionic ectoderm. In *E. schistosa*, it is very probable that the omphalo-placenta exists in the earlier developmental stages and that some of the characteristics of the uterine epithelium of the stenoplacental region are carried over from the yolk-sac placenta.

The description of the stenoplacental region of *E. schistosa* given here will recall to some extent that of Weekes (1929, p. 38) of the placenta of

* "Manche Zellen des Chorinepithels sind stark vakuolisiert. Besonders gegenuber den Spitzen der mutterlichen Villi, an welchen wie gesagt, viel mutterliches Gewebe zerstort ist, treten diese vakuolisierten Zellen haufig auf. Es ist nicht ausgeschlossen, dab hier abgebautes mutterliches Gewebe als Histiotrophe vom Chorionepithel resorbiert wird".

Denisonia superba in the ventral regions of the uterine chambers. The figures (Weekes, 1935, Plate III, Fig. 1) strongly recall the condition found in *E. schistosa*. As her description is too brief and contains no reference to the uterine epithelium of the mid-ventral regions, no complete comparison with *E. schistosa* is possible. A re-examination of the placenta of *D. superba* might be useful and necessary.

The relative functions of the placental regions and their possible bearing on the phylogeny of placental types, call for a few remarks. The function of the placenta is to anchor the developing embryo to the maternal tissues and to supply it with oxygen, water and nutritive material (to a variable extent) and to remove carbon dioxide and excretory materials (Wislocki, 1920). When viviparity is fully established and is accompanied by the diminution of the yolk-content of the ovarian egg—which process does not appear to have commenced in *E. schistosa*—the nutritive function of the placenta becomes steadily greater. Which is the placental region which takes on this function? Previous workers have invariably ascribed a nutritive function to the smaller of the two regions only, where the great development of the maternal and fœtal epithelia makes histiotrophic nutrition possible. Little recognition has been given to the hæmotrophic nutrition which may be inferred to take place almost certainly in the main area of the placenta along with the diffusion of water and respiratory gases. Yet, perhaps, the latter is even more important than the former, being simpler, more direct and less energy-consuming.†

Although the euryplacental region is greater in area than the stenoplacental region, still, its area would have to be much greater if it is to take over the nutrition of the embryo in increasing degrees. Together with the extension of the "endothelio-endothelial" condition to the entire surface, we might expect the area to increase in the course of evolution through folding of the walls. A beginning in this direction has actually been reported in the ground-snakes, *Denisonia superba* and *D. suta* (Weekes, 1929, p. 38-40) whose placenta belongs to type I B (*vide* Table). When the complexity of the ridges and folds increases, the interlocking of maternal and fœtal tissues would become very intimate and the uterine wall would become a much thicker structure (resembling that of the mammals) than is actually found in the viviparous reptiles studied so far. If, in continuation of this

† The elaboration of secretory material by uterine epithelial cells is an energy-consuming process; the breaking down of these substances by the chorionic ectoderm cells and their absorption are equally energy-consuming. This two-fold expenditure of energy is obviated in hæmotrophic nutrition where the simplest carbohydrates and amino-acids diffuse directly from the maternal blood to the fœtal blood.

evolutionary process, the maternal endothelia were to break down, a condition indistinguishable from the hæmochorial placenta of mammals would be reached. Such a line of evolution—admittedly hypothetical—is in harmony with the latest views on the subject of mammalian placentation, which regard the *labyrinthiform hæmochorial placenta* as relatively the more primitive and not the *epitheliochorial placenta* of the ungulates which they regard as not truly primitive but as secondarily specialized (Mossman, 1937, pp. 188–89). The concluding part of the above discussion would perhaps indicate that studies on reptilian allantoplacentation can also contribute to a better understanding of the phylogeny of the advanced types of placenta found in the Mammalia.

Summary

1. The female reproductive system of the sea-snake *Enhydrina schistosa* is described.

2. The disposition of the embryo and the fœtal membranes within the uterine chamber is outlined. The allantois extends into the lower half of the blastocyst and envelopes the yolk-sac also. One pair of umbilical blood-vessels passes directly across the allantoic cavity to reach the outer allantoic membrane.

3. The shell-membrane is entirely absent. The outer surface of the blastocyst is in intimate union with the inner surface of the uterine wall, and placental relationship exists between them. Hence *Enhydrina schistosa* should be ranked as a truly viviparous species.

4. The placental area which covers the entire outer surface of the blastocyst and the contiguous uterine wall, is demarcated into two regions of contrasted characters, for which the names Euryplacental region and Stenoplacental region are proposed.

5. The Euryplacental region which constitutes the main part of the placental area, is characterised by the close attachment of the chorionic epithelium to the uterine epithelium and by the very close approximation of a network of uterine capillaries to a similar network of allantoic capillaries, such approximation being brought about by the shouldering aside of the uterine epithelial cells and the chorionic ectoderm cells. The area is smooth and without foldings. This region doubtless serves for respiratory exchange and for the diffusion of substances in hæmotrophic nutrition.

6. The Stenoplacental region which includes only a small fraction of the placental area, is situated ventrally and has a band-like outline. The

uterine epithelium is specialised for secretion and the chorio-omphalo-allantoic membrane for absorption. The maternal capillaries lie at the base of the uterine epithelial cells and the fœtal capillaries below the chorio-omphalo-allantoic membrane. There is close apposition but no attachment between the surfaces, which, together, are folded to a moderate extent. This region subserves nutrition of a histiotrophic type.

7. The placenta of *E. schistosa* is treated as a new type as it differs widely from the described types, particularly in the characteristics of the stenoplacental region.

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Explanation of Plates

Plate I

Fig. 1. (Photomicrograph) View of the inner surface of the uterine wall, showing the capillary network and the terminations of the small supply-vessels.

Fig. 2. (Photomicrograph) Transverse section through the uterine wall (*ut. w.*) the allanto-chorion and the inner allantoic membranes (*all. ch.*) in the euryplacental region. The same section is sketched in Text-Fig. 6.

FIG. 3. (Photomicrograph) Transverse section through the uterine wall and the allanto-chorion in the euryplacental region, showing the chorionic ectoderm cells pulled out into conical projections as a result of forcible separation from the uterine wall. *e.c.*, ectoderm cells.

PLATE II

FIG. 4. (Photograph) Two detached uterine chambers from *Enhydrina* showing clearly the blood-vessels on the uterine wall. *o.f.*, oviducal funnel.

FIG. 5. (Photomicrograph) Transverse section through the entire width of the stenoplacental region (*S.P.R.*). The same section is sketched in Text-Fig. 8. A portion of the euryplacental region (*G.P.R.*) also is seen because the complete uterine wall together with the allanto-chorion was rolled up and sectioned.

FIG. 6. (Photomicrograph) A small area from the section in Photo. 5 at a higher magnification, showing the allantoic endoderm stopping short on one side.

all.b.v.,	allantoic blood-vessel	*m.d.*,	dense layer of mesodermal tissue
all.e.,	allantoic endoderm	*o.all.m.*,	outer allantoic membrane
i.all.m.,	inner allantoic membrane	*y.e.*,	yolk endoderm

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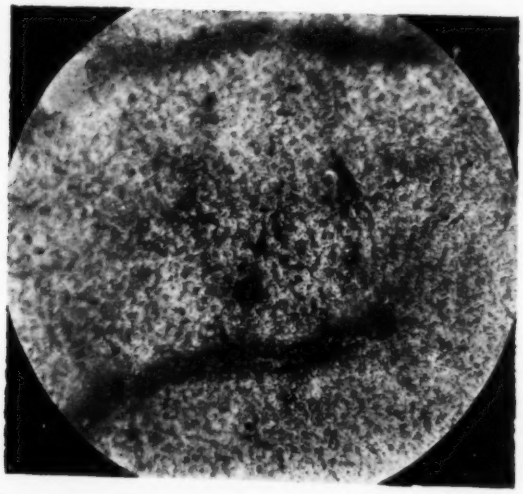

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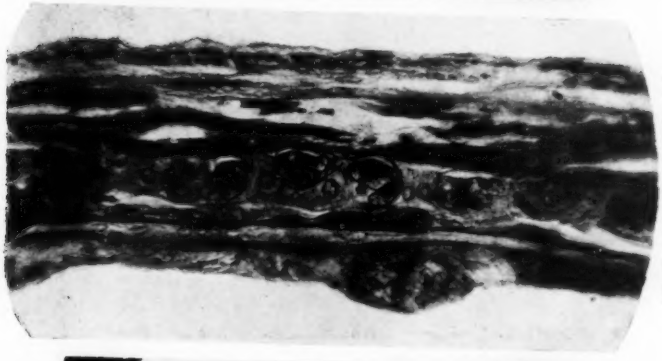

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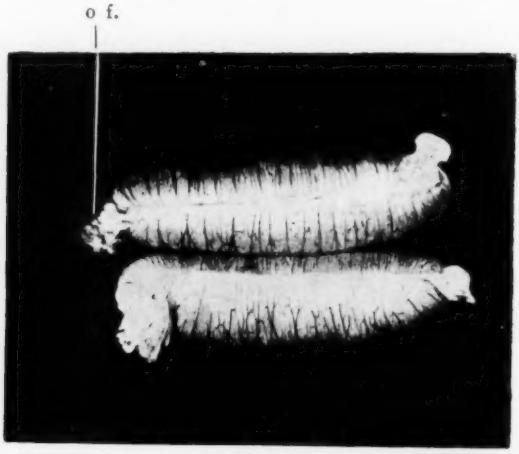

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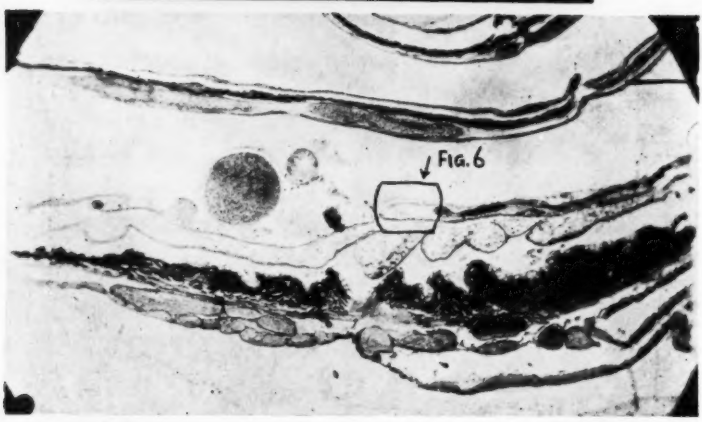

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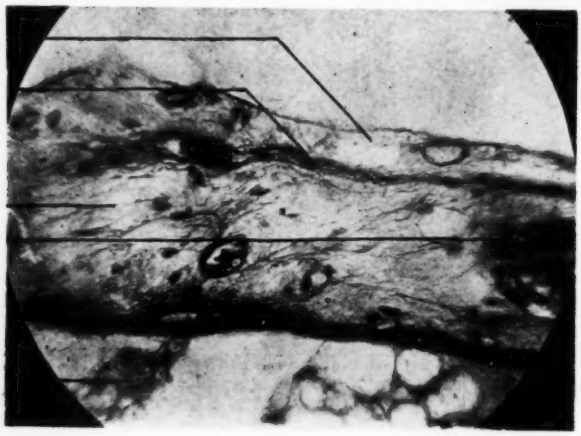

6

POLYGALACTURONASE ACTIVITY OF *ASPERGILLI*

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Introduction

Polygalacturonase (PG) (Pectinase) catalyzes the hydrolysis of 1, 4-α-glycosidic linkage of anhydrogalacturonic acid units in pectic acid. In fungi PG is always associated with pectinesterase (PE)[1,2] and the separation of these two enzymes has been effected recently by use of cation exchange resin[3] or by acid treatments followed by precipitation with ammonium sulphate between treatments.[4] Although qualitatively the occurrence of PG in plant materials is known,[1] its elaboration by phytopathological micro-organisms as well as non-parasitic fungi has been studied more extensively.[5–11] Reports are however not always in agreement regarding the optimum cultural conditions for PG secretion.

This study relates to PG elaboration by three strains of *Aspergilli* isolated in the laboratory and the preparation and properties of an active PG product.

Experimental

Measurement of Activity

PG activity was followed by determining increase in reducing power of pectin solution.[12] 100 c.c. of 1 per cent. solution of citrus pectin (analysing to 1·15 per cent. ash, 75·20 per cent. uronic acid[13] and 4·18 per cent. CH_3O[14] on dry basis) was adjusted to pH 3·6–3·7, reported optimum for the enzyme[15,16] and 10 c.c. of the enzyme extract was added and incubated at 37° C. for 48 hours after addition of a few drops of toluene. The reducing values were determined by Willstätter and Schudel's hypoiodite method.[17] Blank determinations were carried out using 10 c.c. of the inactivated enzyme extract. The amount of the galacturonic acid corresponding to the thiosulphate values were read off directly from Kertesz' data[18] which were suitably standardized. The activity of PG is expressed as the percentage of galacturonic acid liberated on its yield as obtained by decarboxylation with 12 per cent. hydrochloric acid according to Dickson, *et al.*[13]

Although pectic acid is the ideal substrate for PG action, the method as employed, using a low ester pectin, was found quite satisfactory in

practice. The hydrolysis of pectinic acid to galacturonic acid by PG alone is not complete, but the presence invariably of associated PE with PG would release the esterified portion for PG action. The rate of liberation of galacturonic acid would be affected by the relative proportions of PG and PE in different fungi, but it was observed that, under the conditions of hydrolysis, the reducing value was maximum after 24–48 hours, a further 8–10 days period resulting only in 3–5 per cent. increase in the different cases.

Isolation and Culture of Organisms

A dilute glucose-Czapek-Dox medium containing 1 per cent. pectin was exposed to air. At the end of 4–5 days, eight different strains of moulds were obtained; and these were isolated in pure forms by "agar-dilution method".[19] Out of eight strains (*Aspergilli*, *Penicillia* and *Rhizopus*) only three of *Aspergilli* designated here as Nos. I, III and V were pectolytic as observed by partial liquefaction in sodium pectate-containing solid media.[20] The stock cultures were made and maintained on Czapek-Dox agar slants. They were subcultured thrice before transferring to liquid media.

Fifty c.c. of Czapek-Dox medium were inoculated after sterilization with 0·5 c.c. of a heavy suspension of spores of the selected strain of fungus and incubated at room temperature. At the end of 4–5 days, 2 c.c. of toluene were added to each flask and the fungal mycelia were collected on a tared filtered paper and dried to constant weight at 100° C. PG activity was determined in the culture filtrate (Table I).

TABLE I

PG Activity in Liquid Media

Moulds	*Aspergillus*					*Penicillium*		*Rhizopus*
	I	II	III	IV	V	I	II	I
Dry weight of mycelium (mg.)	387	342	258	396	432	463	401	312
PG activity ..	7·4	4·5	13·2	3·5	12·4	4·4	..	..

Only *Aspergilli* I, II, III and V were used for further studies. These have been identified as *A. luchuensis* Inui, *A. flavus* Link and *A. niger* van Tiegh respectively. *A. luchuensis* Inui was a strain with longer conidiophores and larger vesicles than is typical for the species. The use of pectin has been

advocated for stimulating PG formation.[6] Supplementation of culture solution with 1 per cent. pectin stimulated only the growth of the organism without significant enzyme secretion. A time study of the growth of mould, PG activity and changes in pH and reducing values with two of the strains in pectin-enriched media is given in Figs. 1 and 2.

Growth was rapid initially but slowed down after 5–7 days. PG activity was maximum after 4th day of incubation and diminished gradually thereafter. There was parallelism, especially with *A. flavus*, in reducing power by glycosidic hydrolysis of the pectin molecule; its subsequent decrease is due possibly to utilization of galacturonic acid by the mould for its growth. The comparatively slow increase in reducing value with

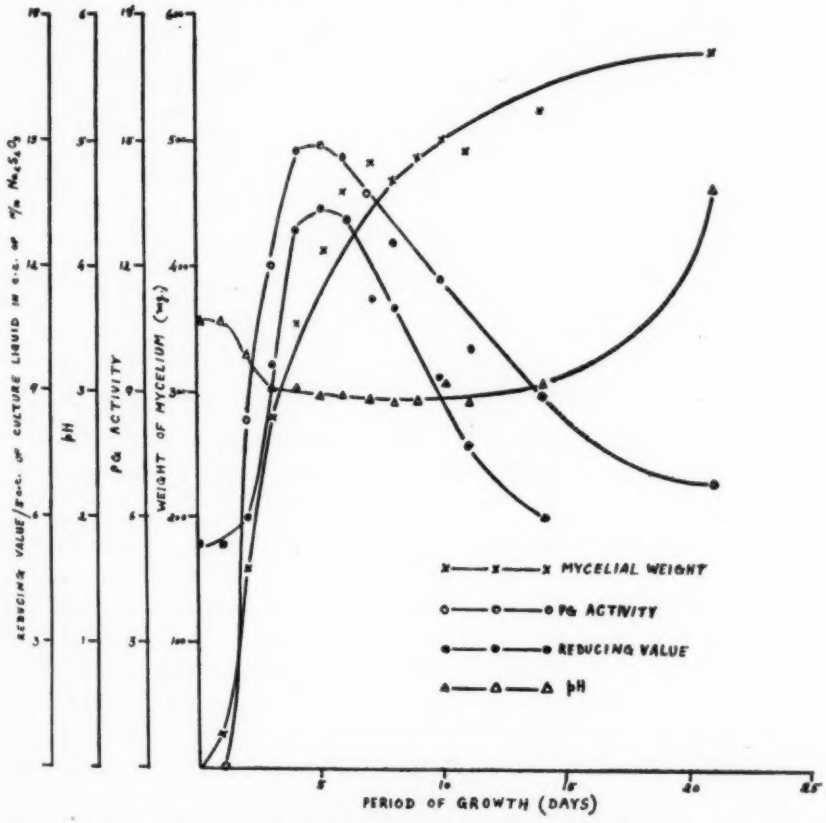

FIG. 1. Changes in Mycelial Weight, PG Activity, Reducing Value and pH during Growth of *A. flavus*

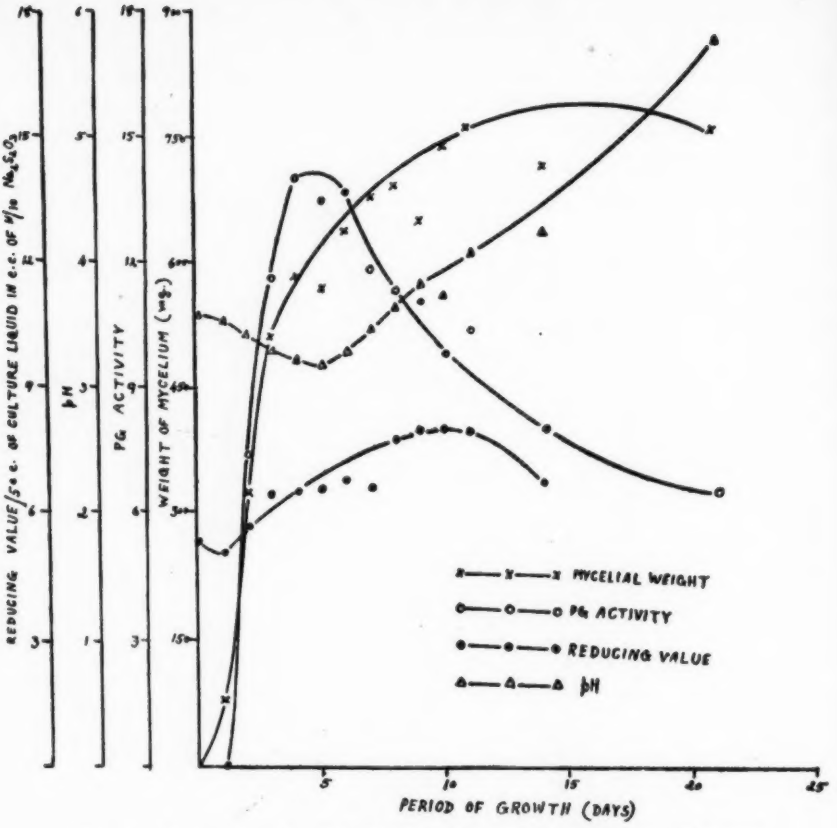

FIG. 2. Changes in Mycelial Weight, PG Activity, Reducing Value and pH during Growth of *A. niger*

A. niger would suggest that the organism utilized galacturonic acid more rapidly, its accumulation in the medium therefore not being well marked. The initial small drop in reducing value is due to the utilization of glucose of the medium by the mould. After an initial drop in pH, the value rises gradually in case of *A. niger*, while with *A. flavus* a more or less steady pH is maintained for a prolonged period. The liberation of galacturonic acid may account in part for the initial drop in pH in both cases.

Maximum elaboration of enzyme took place, when the pH of the medium was between 3·0 and 4·5. Higher pH values were detrimental to formation of PG but mycelial weight was comparatively less affected (Fig. 3). It should be stated here that formation of PG was mostly exocellular with all

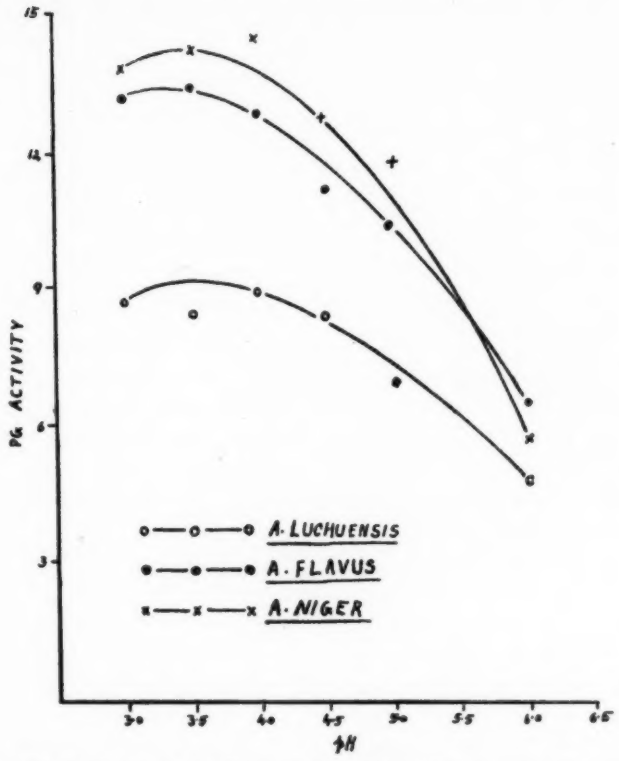

FIG. 3. Effect of pH of Medium on PG Elaboration at end of Four Days

the strains, the mycelial extracts (aqueous or 5 per cent. glycerine) showing negligible activity.

Use of Solid Media

Wheat bran is a complete habitat for growth of moulds and proved very satisfactory as medium for production of mould amylases.[21–23] In the following experiments 10 g. lots of wheat bran moistened with 15 c.c. of water were sterilized (at 20 lbs. pressure for 30 minutes) and inoculated after cooling with 0·5 c.c. of the spore suspensions. At the end of every 24 hours, one flask was taken for extraction of the enzyme. Thirty five c.c. of water were added to the flask and kept overnight for autolysis after addition of toluene. The extract was squeezed out, made up to volume, centrifuged and used for determination of PG activity (Table II).

TABLE II. *Use of Wheat Bran as Medium for Growth*

Age of culture (days)	PG activity		
	A. luchuensis	*A. flavus*	*A. niger*
2	2·38	7·00	12·8
3	7·02	10·42	17·11
4	10·76	13·13	22·11
5	8·74	11·68	16·77
6	7·14	9·40	15·97

PG activity was better with wheat bran than when synthetic liquid medium was employed. The enzyme content was maximum after 4 days which was the period of harvesting in subsequent experiments. The use of 5 per cent. glycerine for extraction of the mouldy bran resulted in 90–180 per cent. greater recovery of the enzyme (Table III).

TABLE III. *Extraction of Enzyme by Aqueous Glycerine*

	PG activity		
	A. luchuensis	*A flavus*	*A. niger*
Water extract ..	10·76	13·13	22·11
Glycerine extract	19·83	37·60	43·84

Addition of pectin had practically no effect on production of PG but supplementation with hydrolyzed casein resulted in definite stimulation (Table IV).

TABLE IV. *Effect of Addition of Pectin and Tryptic Digest of Casein Hydrolysate* to Wheat Bran*

Supplement	PG activity		
	A. luchuensis	*A. flavus*	*A. niger*
None ..	19·0	37·9	42·6
1% Pectin ..	18·9	38·3	41·0
0·1% Casein hydrolysate	34·0	63·9	67·1

* Aminoxyl: Raptakos, Brett and Co. Ltd., Bombay.

Preparation and Partial Purification of PG Enzyme

Wheat bran containing 0·1 per cent. enzymic casein hydrolysate was inoculated with spore suspensions of each of three *Aspergilli* organisms. At end of 4 days, the material was extracted with 5 per cent. glycerine, strained and centrifuged at 3,000 r.p.m. for 5 minutes. Alcohol was added to the centrifugate to a concentration of 80 per cent. The precipitate obtained after centrifuging was dried in a vacuum oven at 40° C. The activities of the alcohol precipitates were 9·2, 15·9 and 24·0 per cent. (expressed as per cent. on maximum yield of galacturonic acid) for *A. luchuensis*, *A. flavus* and *A. niger* respectively.

During precipitation with alcohol, therefore, there was destruction of enzyme to the extent of 60–70 per cent. Fractional precipitation with increasing concentrations of ammonium sulphate resulted in comparatively smaller loss (Fig. 4); the enzyme is precipitated mostly between 40–60 per cent. concentration of ammonium sulphate; precipitates obtained at lower concentrations of the salt consisted mainly of non-enzymic matter.

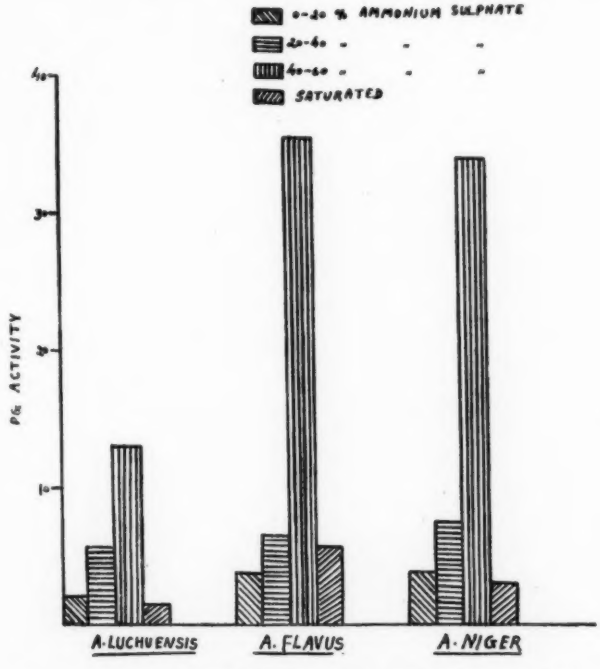

FIG. 4. Fractional Precipitation with Ammonium Sulphate

Using *A. niger*, a partially purified PG preparation was obtained by precipitating the enzyme extract from 500 g. of wheat bran with 60 per cent. concentration of ammonium sulphate after discarding the precipitate obtained at 20 per cent. salt concentration. The yield was about 19 g. and it had approximately 55–60 per cent. of the original activity. More or less similar results were obtained with *A. luchuensis* and *A. flavus*. The preparation was dark brown in colour and was further purified by a second salting out with ammonium sulphate in the same manner as above when a light brownish coloured product was obtained retaining about 40 per cent. of the original activity. The product was dried *in vacuo* without loss in activity. The yield was 12 g.

The activity of this preparation (10 c.c. of 0·5 per cent.) was studied with regard to time and pH using 100 c.c. of 1 per cent. citrus pectin as substrate (Fig. 5). At pH 3·7 the activity increased with time, at first

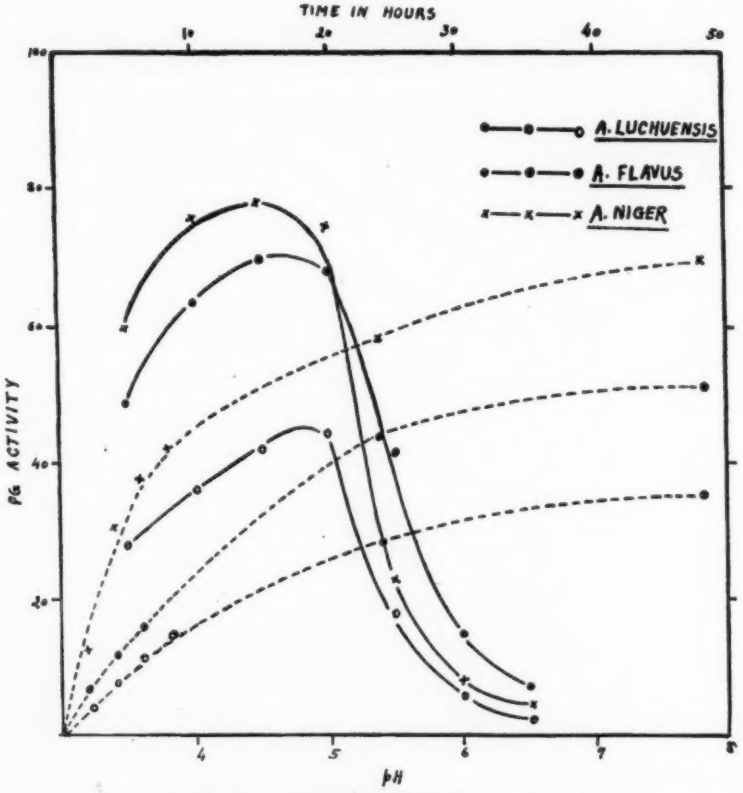

FIG. 5. Variation of PG Activity with Time and pH

rapidly, during 12–24 hours; further hydrolysis was slow and continued for 8–10 days. The activity at pH 3·7 was considerable; it increased rapidly with a maximum between 4 and 5; subsequent drop was also rapid.[8]

Discussion

PG formation has been studied in case of a few *Aspergilli*, especially *A. niger* and *A. oryzæ*. In the studies reported here, all the five different *Aspergilli* isolated in this laboratory were pectolytic to varying extents. Of these, *A. luchuensis* Inui, *A. flavus* Link and *A. niger* van Tiegh were rich in PG activity, the enzyme being mostly exocellular. The production efficiencies of *A. flavus* and *A. niger* compared very favourably with those reported for other organisms,[9,10] while that of *A. luchuensis* was comparatively poor. The time of maximum elaboration of enzyme with all these organisms was considerably shorter than has been obtained hitherto.

Wheat bran was a better substrate than the synthetic medium for PG elaboration by the organisms. While this may be due to its better texture, promoting greater aeration, it would seem that its nutrient status has also contributed to its superiority. It is significant that supplementation with casein hydrolysate has resulted in greater PG activity thus suggesting that nitrogen source is important.

PG is shown as an adaptive enzyme by many workers,[6,9] its formation being stimulated by specific substrate additions. The addition of pectin did not however stimulate the formation of PG in the *Aspergilli* studied here and would therefore emphasise the non-adaptive nature of PG as observed by Gaumann and Bohni[10]; evidently this property varies with different strains.

Summary

1. Three strains of *Aspergilli* having good PG activity were isolated.

2. The conditions for maximum elaboration of PG were studied using synthetic liquid media as well as wheat bran.

(i) The enzyme content was maximum after 4 days of growth; pH below 5 favoured the formation of enzyme.

(ii) Wheat bran was a better substrate than synthetic liquid media; aqueous glycerine was very satisfactory for extraction of the enzyme from wheat bran medium.

3. Partially purified PG preparations were obtained by precipitating enzyme at 60 per cent. concentration of ammonium sulphate after discarding

the precipitate at 20 per cent. salt concentration: alcohol inactivated the enzyme to a great extent.

4. The optimum pH for activity with citrus pectin as substrate was about 4–5; hydrolysis was rapid during first 12–24 hours and decreased thereafter during a further 8–10 days period.

Acknowledgment

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SOME ASPECTS OF REGENERATION IN LEAF CUTTINGS OF TOMATO

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Regeneration by stem cuttings, in the tomato plant, is well known, and through leaf and leaflet cuttings, though not common, has also been pointed out for some time past (Isbell[2]). It is striking to note that roots in Isbell's experiment were readily regenerated by both the leaf and the leaflet cuttings, but shoots on the other hand could be regenerated only by the leaf cuttings. Most of the roots, according to this author, arise from the callus region formed at the base of the petioles or petiolules, but shoots have been shown to have only a terminal dominancy, appearing in the axils of leaflets, which are terminally located. As in the case of potato, root, tuber and shoot altogether could arise from the same point at the leaf base, the observations of Isbell on leaf cuttings of tomato appeared far fetched. In order, thus, to throw more light on the possibilities of regeneration in the tomato plants, the experiments on leaf cuttings were repeated.

A number of well-developed leaves from healthy tomato plants (*Lycopersicum esculentum* var. Pocha large red) were carefully detached on December 28th, 1948, in evening hours, when accumulation of food through photosynthetic process had reached its maximum for the day. They were immediately placed in beakers over water, a part of the petiole dipping.

Under the laboratory conditions* (temperature 22 $\pm$ 1° C.), rooting on petioles in some cases and on both petioles and petiolules in others occurred after 13 days of planting (Figs. 1 and 2). In some cases small tubercle-like swellings (Fig. 2-S) also appeared at the axils of certain leaflets. These outgrowths were obviously the essentials of shoot regenerates.

* Banaras is situated in latitude 25° 19′ N. and longitude 83° 03′ E., at a height of 267 feet above mean sea-level.

A careful examination of leaf cuttings showed (1) that root regenerates did not limit themselves to the callus part of the petioles but extended to considerable distances, both on the peticle and the petiolules, and (2) shoot regenerates similarly did not confine themselves to the terminal regions of the leaves. These findings (Figs. 1 and 2) stand in contrast to those of Isbell, though they do not necessarily refute them. Perhaps, Isbell's findings were of a limited nature and held good for the conditions in which he conducted his experiments. In the present investigation, cuttings were regenerated in tap water, which was more freely available, against the one in sand substratum used by Isbell.

It seems likely that regeneration capacities in the tomato plant for either the shoot or the root do not limit themselves to any particular regions or organ of the plants; but extend themselves to a whole range wherever conditions for regeneration exist. When leaf cuttings of potato could produce all sorts of regenerates (root, shoot and tuber) at one and the same point (*cf.* Kupfer,[3] and Isbell[2]), when both root and shoot regenerates could be produced in the same notch in *Bryophyllum calycinum* (Singh and Mehra[4]), and when identical regenerates could be found in animals as widely separated as coelenterates, flat-worms, segmented worms, and chordates (Curtis[1]), such a possibility in the case of tomato plants, which are highly susceptible in this repect, is not far fetched. In fact, rooting has been induced in almost all plant organs including leaves, flowers, petals, peduncles and petioles of several species of plants (Zimmerman[5]) through the application of growth substances, which well indicates that all parts and organs of plants are susceptible to regeneration if adequate conditions are available.

Conclusions

(1) Rooting in leaf cuttings is not confined to callus region of the petiole or petiolules but can extend to considerable distances if conditions are favourable.

(2) Regeneration in leaf cuttings of tomato is apparently not different than found to occur in other plants.

(3) Terminal dominancy in tomato as suggested by Isbell cannot be taken as a rule as both shoot and root could arise by the same organ irrespective of position and perhaps at the same point.

(4) In case of tomato, perhaps, full regenerates could also be produced from leaflets alike leaves.

1 2

FIGS. 1 and 2.—Root regenerates in leaf cuttings of tomato (basal part of the petiole cut off to accommodate in the photograph)—(1) Showing regenerates on the petiole extending beyond the limits of callus and (2) Regenerates on the petiole and petiolules similarly extending over considerable distances. The tubercle-like swelling shown in Fig. 2 by letter 'S' as located in one of the leaflet axil, denotes the shoot regenerate.

1.

2.

3.

4.

5.

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MINOR ELEMENTS IN SOIL AND THEIR EFFECT ON FORAGE AND FEEDING CATTLE WITH SPECIAL REFERENCE TO THEIR SIGNIFICANCE AS TRACERS OF EVOLUTION OF SOME COMPLEX ORGANIC STRUCTURES: AS DETERMINED BY APPLICATION OF GOLDSCHMIDT'S LAW

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§1. Insecure Foundation of Present Knowledge of Interrelation of Trace Elements

There are elements like Fe and Al which are required in ' minor ' proportions for plants but nevertheless present in major proportions in soils, while the reverse is true for elements like N. This shows that the entry of those elements into the plants is not entirely governed by the law of mass action. There are elements like Co and I which are present in forage simply because they are available in the soil, serving no essential purpose for themselves although essential for feeding cattle. This means that this entry is neither exclusively governed by any known biological function of these elements in the absorbing organism. There are also elements like Se and F which though not so poisonous to plants are toxic to animals. Mn, Fe and Cu are essential to both plants and animals, while B is essential only for plants. Mo, on the other hand, although essential for some plants, soon reaches toxic limits for animals. K and Mg are present in major quantities in plants, while their quantitative importance is taken up by Na and Fe in animals. There is a curious relation between Mo causing ' teart ' disease in animals, and Co causing ' pining ' in sheep observed in eastern Rose-Shire. There are some running down or wasting diseases of animals which were cured by Cu and Co although the herbage showed no such deficiency. Much information in this regard is desirable that can show the causes for these curious interrelationships and antagonisms between the different elements. The above facts only show that the quantity which enters the organism is not exactly according to what is available in the medium nor is it entirely limited by any specific function in that organism. In the words of Watson

and Smith (1946) " there seems to be a curious interaction or antagonism between the trace elements and consequently the indiscriminate use of mixture should be avoided until our information is better founded ".

There is need therefore to review the position from the point of view of newer chemistry of those elements based on ionic or atomic radii and covalency or co-ordination theory which are really fundamental and to see if these considerations can co-ordinate and put on a better foundation at least some of the seemingly conflicting facts noted above regarding the interaction and function of the different elements. Baudisch (1945) applied concepts of magneto-chemistry in early attempts at understanding the biological functions of trace-elements but the concepts of crystal chemistry do not seem to have been applied to this field so far.

§2. Possible Steps in the Evolution of Some of the Essential Complex Organic Structures

Comparing for example the esssntial structure of (1) the respiratory pigments like the cytochromes, (2) hæmoglobin and (3) the chlorophylls, it is found that they are all metal derivatives of porphyrins to which are co-ordinated two more nitrogen atoms of a protein, with similar X-ray patterns and differences only in side chains.* The iron in the universally present respiratory pigments like the cytochromes, which are the most primary structures in the embryo and evolution (Barcroft), can change from the ferrous to ferric or *vice versa*, the substituents in the poryphyrins and the proteins being such that the corresponding change in the valencies and double bonds can be reversibly communicated from one end of the molecule to another and finally from one substrate to another. The evolution of other substances like hæmoglobin or chlorophylls from these primary structures can be pictured in two important steps, either firstly a metallic substitution and secondly then by a change in the side chains, or in a way in which the order of these two steps is reversed. It is proposed to show in this discussion that the first of these two alternatives is more natural as in that way the second of the two steps can be shown as a consequence of the first which in its turn can be taken to conform to the well-known law in geochemistry established by the pioneering work of Goldschmidt (1944) and his school. This deals with the replacement of one ion or atom by another in the framework of minerals and clays, and postulates that one ion or atom can replace another only if their radii agree within 10 per cent. of

* One difference between cytochrome and the rest is that it has the iminozole N of the protein satisfying the co-ordination number of the metal while the others have not got them in a position to satisfy it.

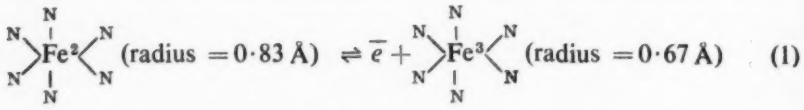
each other. These premises, it will be shown, are sufficient to explain many of the peculiarities and relationships between the trace elements noted in Section I.

§3. Evidence for the Ionic Replacements in the Build Up of Complex Organic Substances

In the experiments of Rama Moorthy and Desai (1946) an iron injection into one half of a chlorotic tobacco leaf showing symptoms due to an iron deficiency, leaving the other half as the strictest possible control resulted in the quick appearance of colour and chlorophyll in that half as was to be expected, accompanied curiously enough by a significant decrease in the absolute Mn content (as for example from 31·3 to 4·3 part per million on dry matter basis) following the increase in the iron content due to injection. This is (direct) evidence for the rôle of ionic interchanges in the build up of chlorophyll by the plant.

§4. Evolution of Hæmoglobin and the Function of Cu and Co in Curing Anæmia

The cytochromes may be pictured as in which the iron is held by co-valency from 4 N atoms of the porphyrin on the same plane with two iminozole N atoms of the protein on either side (top and bottom). It can only change from ferrous to ferric or *vice versa* under the stimulation of the substrate, but it cannot add additive compounds as all the six co-ordination bonds are strongly held by six N atoms (Theorell, 1941). There is also an equilibrium between the ferrous and ferric compound, by reversibly changing which it acts as donor or receptor in the oxidation-reduction reactions connected with respiration. The side chains and double bonds are, however, such that they reversibly react to the consequences following this oxidation and reduction of iron and this may simply be represented as follows:—

 (radius $= 0{\cdot}83$ Å) $\rightleftharpoons \bar{e} +$ (radius $= 0{\cdot}67$ Å) (1)

It will be seen according to Goldschmidt's law applied to biology that Fe^2 with radius 0·83 Å can be replaced by Mg^2 (0·78), Mn^2 (0·91), Zn^2 (0·83), Cu^2 (0·80) and Co^2 (0·82) while Fe^3 (0·67) can be replaced by Mn^3 (0·70), Co^3 (0·65) and Mo^4 (0·68).

Suppose that in the course of evolution or development of the embryo, the respiratory cytochromes come into contact with solutions containing Cu^{2}. The latter will replace Fe^{2} of the cytochrome and the effect is to shift the equilibrium in equation (1) to the left and then get continuously replaced by Cu^{2} till the reduction of Cu^{2} complex to the Cu^{+1} complex opposes the formation of Fe^{2} complex from the Fe^{3} complex.† The effect is the attainment of a thermodynamic equilibrium between the two couples.

If such a state of affairs happens to the copper prophyrin formed by substitution, there will be a further increase in the size of the central atom (Cu^{+1} radius 0·96) creating a force throwing away the N atoms, primarily affecting the relatively weaker bonds attached to the iminazole nitrogen which can be rotated in such a way as to allow of other atoms coming in for satisfying the co-ordination number of the centre atom. Thus the first effect of substitution by Cu in a cytochrome molecule is to orient the iminazole nitrogen bonds so far away that the new molecule can form additive compounds with other molecules (like O_2 in the case of hæmoglobin and CO_2 in the case of chlorophylls). This may be the reason for the essentiality of copper both for plants and animals. The other effect is that the molecule which could adjust to a changing valency of 2 to 3 of the central atom has to adjust to a further reduction in the valency of the central atom to 1. This after the lapse of some time obviously means certain changes and rearrangement of the double bonds and side chains and hence the molecule may not be able to retain that reversibly labile nature it possessed in the cytochrome molecule. If now Co^{2} (0·82) is also present, it can replace Cu^{2} (0·80) which is in dynamic equilibrium between Fe^{2} complex on one hand and Cu^{+1} complex on the other and prevent the irreversible changes associated with copper going too far. It may be mentioned here that this Cu^{2} complex is not exactly identical with that first formed from cytochrome but is the one with the necessary strain resulting from its dynamic equilibrium with its own couple containing the cuprous complex. Subsequently this Co^{2} can be replaced by Fe^{2} to form the hæmoglobin which can unlike the parent cytochrome form additive compounds with O_2 without undergoing oxidation but resembles the cytochrome more than the chlorophyll does. It may be that this Co complex is a stable intermediate and can be isolated. Smith (1945) isolated a Co containing anti-pernecious anæmia factor whose constitution is not yet established.

† One of the chief factors determining the frequency and extent of the different changes is the oxidation and reduction potential involved.

§5. Function of Mo in Plants and Animals and Its Antagonism with Copper and Cobalt

Suppose instead of Cu and Co as above, there was Mo in solution ....Mo^4 (0·68) can replace Fe^3 instead of Fe^2 and unlike in the case of Cu^2, the equilibrium in equation (1) of the cytochrome will shift to the right. Mo^4 has a higher co-ordination number of 8, a higher valency, smaller diameter and greater affinity for nitrogen compared to iron. The result of substitution by Mo is that the two valencies from 4-N atoms of the porphyrin mesomerically holding Fe have to accommodate Mo with four valencies and therefore such a strain in the molecule is induced that what was previously functioning as a respiratory pigment has to change the nature and reversibility in its side chains and bonds and the molecule begins to fix nitrogen. This explains the essentiality of Mo (Burk; Bortells, 1939) for biological nitrogen fixation. As the change induced by Cu and Co which are necessary for hæmoglobin formation is just opposite in direction compared to the change induced by Mo, this gives a clue as to how an excess of Mo in the feed can cause anæmia or 'teart' disease noted in sheep in Somerset and Worcester and how Cu acts as corrective for this purpose.

The antoganism between copper and lead found in the Swayback disease of animals is however slightly different in nature. Pb^4 with a radius of 0·84 A can replace Fe in competition with Cu^2; because of its greater valency and co-ordination number Pb^4 can produce at least part of the effects described under Mo^4 in the matter of fixing additive compounds. The effects however do not last long as on reduction Pb^4 changes to Pb^2 with a diameter of 1·32 Å and because of the accompanying expansive force described under Cu^+, the capacity for additive compound formation is enhanced. But this takes the molecule to a situation where this Pb^2 (1·32) can be easily exchanged by the mobile monovalent K ($\gamma = 1{\cdot}33$ Å) with its greater solubility and capacity to ionise, in addition to the rearrangements made necessary when the side chains and bands are to adjust to the new valency of the central atom. Hence there is a great tendency for the molecule to decompose. These effects can however be counteracted by Cu which not only can initiate its own chain of reactions in competition with Pb^4 but can also replace Pb^4 from the complex even in the initial stages using the replaced Pb^4 for oxidising any Cu^+ in the medium. Thus it is that Cu is cure for the Pb induced swayback.

§6. Building up of the Chlorophyll and the Function of Mn and Mg in it

If after the action of Cu, Mn^2 (0·91) be present it can replace Cu^{+1} before the side chains and double bonds lose their accommodating power for the

changing valencies of the central atom, although due to the changes associated with copper, liability now can be expected only under external excitation (like the action of light) and at this stage this ability could be kept up by the action of Mn which can change valencies just like the Fe atom. If at this stage Mg^2 (0·79) replaces Mn^3, the result even under external stimulation is that, although the side chains and double bonds do adjust to a possible reduction in the valency of the central atom, they find that the new central atom (Mg) can no longer change its valency and have therefore to reduce the additive compounds like Co_2 held by this Mg in co-ordination. This may be how chlorophyll gets its functions and abilities compared to the parent cytochromes.

If, however, excess of Mn be present, it might prevent Cu doing its part as the oxidation potentials involved in the reactions $Mn^2 = Mn^3 + \bar{e}$, $Cu^+ = Cu^2 + \bar{e}$, are respectively $-1{\cdot}51$ and $-0{\cdot}167$ volts. This can be remedied by more iron, which may form more of the cytochrome to start with and excreting the un-utilisable Mn compounds.

§7. Sulphur–Selenium Relationship

Biologically sulphur is an essential constituent of proteins and hair especially. If, however, Se which can easily replace S by virtue of their chemical relationship, ionic or atomic radii, substitutes S in part, it will break the S-cycle by virtue of its greater metallic nature and will therefore be biologically harmful, resulting in bad wool production. Se is not so toxic for plants either because the metabolism of S, producing wool, is not important in plants or that in plants, Mo^6 or Mn^4 or B or other element specifically present might counteract the action of Se in a way at present not so clear.

Discussion and Conclusion

The picture of the complex molecules in the living cell like the cytochromes herein presented is that they are arranged and act as sieves, the walls of which are made of nitrogen atoms, the thickness of the walls being made up of the porphyrin and the protein. Hence the importance of ionic radii for the admission and retention of the different metatlic ions in these sieves. Although no base exchange of the type shown by Gedroitz in soils and clays is indicated which is mainly a surface phenomenon, what is expected is only the ionic interchange of the type shown by Goldschmidt in the minerals formed out of the magma. This interchange which is a phenomenon of the interior is expected to be valid only a short while during and after the formation of the original molecular pattern. That is why the metallic porphyrins extracted from the living cell do not so easily exchange

their metal ions because during extraction they lose their original arrangement in the living cell. There is however some evidence on the effect of the substitution of Mn for Fe in the hæmins (Theorell, 1945). But from analogy with the extracted metallo porphyrins, those in the living cells cannot be expected to be so slow in exchange. For once the principle of exchanges is conceded, the frequency of exchanges with Fe, Cu, Mn and Mg, etc., needed for example in the formation of chloryphyll in the plant must be as frequent as the formation of chlorophyll itself in the plant, which must be sufficiently high indeed !

The frequency of this ionic interchange is determined by (1) energy considerations, (2) agreement with ionic or atomic radii and (3) periods of time course of plant reactions or periods of " Chemical-physical sensitivity ". To these Prof. H. Lundegardh in a private communication suggests the " addition of obstacles founded in the complicated structure of the protoplasm " resulting in the selectivity or " favouring the absorption of certain ions and impeding others, although the barriers break in a pathological way when these elements are present in toxic concentrations. The importance of ionic interchange " in exchange processes had been emphasised by Lundegardh (1946). The idea presented in this paper of a combined action of different heavy metal atoms of similar dimensions in certain physiological process gets experimental support in a very recent article published subsequent to writing of this paper on the activation of phospho-glucomutase by metal ions wherein the experimental proof for the combined action of two metal atoms for this enzyme reaction is provided by Stickland (1949). Thus his theory not only explains and correlates a number of experimental facts regarding ionic antagonism and their essentiality for certain important biological reactions but also provides a possible link in the probable evolution of some of the essential complex organic structures in the living organism. This theory also indicates future possibilities of experimentation, as well as possible extension to cover other metallo complexes in the organism other than porphyrins. For example, Pb is expected to supplement Mo for nitrogen fixation where as Cu or Cu and Co are expected to hinder or antagonise the effect of Mo.

Goldschmidt's law also states that amongst those metals that so agree in radii within 10–15% of each other, those that have a greater bonding strength get a preference over the rest, concentrations remaining the same. This bonding strength increases with the valency and decreases with the radius of the ion. The normal permeability of an ion in an organism is therefore determined by the condition that its radius should agree within

10–15% of the space in the lattice of the complex organic structures in the organism that are capable of accommodating a metal ion, while the avidity with which it is absorbed depends on the bonding strength of the ion and its concentration in the medium. These then should determine whatever specificity that is attached to the biological distribution of these trace-elements along with the fact that the organism should be able to perpetuate itself in the presence of that ion in order to show its distribution, and for the normal type of co-ordination, this avidity gives rise to the normal Mellor and Maley series of ionic preferences.

Summary

There are many gaps in our knowledge of the biological interrelationships and antagonisms of the trace elements. For example, there are certain animal deficiency diseases of Cu and Co which plant analyses for these elements do not reveal unless there is an understanding of the elements which antagonise them and of those related.

The application of Goldschmidt's law to the replacements of atoms and ions in complex crystal structures (in the form that such replacements are possible only when the size of the atoms concerned agree within 10% of each other) coupled with a knowledge of the changes in valency and oxidation reduction systems involved, has been shown in this paper to elucidate many of these relationships and antagonisms.

The essentiality of Cu and Co for hæmoglobin formation, the cause of anæmia due their deficiencies and how they are antagonised by Mo are shown, as also the essentiality of Mo for N fixation and the antagonism between Cu and Pb in the sway-back diseases. The essentiality of Fe, Cu, Mn and Mg for chloryphyl formation and the possible steps in the evolution of chloryphyl and hæmoglobin from cytochrome by the combined action of intermediary metal atoms have been traced along with the biological antagonism between Se and S. A consequence of this is the greater importance of Mo for leguminous plants compared to others with a tendency for the deficiency in cattle of Cu in countries where legumes and the like are extensively grown.

Acknowledgement

My thanks are due to Profs. Sir C. N. Hinshelwood (Oxford), and B. Viswanath and Drs. B. P. Pal, J. N. Mukherjee, S. V. Desai, S. P. Ray-Chaudhuri, N. P. Datta, A. Eden (Cambridge), Tamahne, Dakshinamurty and N. B. Das for their kind interest in this work, to Prof. H. Lundegardh (Sweden) for his kind comments and suggestions referred to in the discussion

and to Dr. Robert Hill (School of Biochemistry, Cambridge) for reading the manuscript and suggesting improvements.

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CHROMOSOME EVOLUTION IN WILD POPULATIONS OF ACRIDIDÆ

Part I. On the Occurrence of a Supernumerary Chromosome in *Aiolopus* sp.

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(Communicated by I. Banerji, D.Sc., F.A.Sc., F.N.I., F.L.S.)

Introduction

The study of the spontaneous variability of the chromosomes in the natural population is a prerequisite for a proper understanding of the mechanism of chromosome evolution. It demands the recording of cytological data from a large number of specimens, collected and examined individually, from various localities. In the past, similar studies in Acrididæ (*e. g.*, Carothers, 1917; King, 1923; Helwig, 1929) have yielded useful and interesting data. The literature on the subject has been recently reviewed by White (1945) where he has pointed out various lacunæ in our knowledge, most of which are due to lack of sufficient quantitative data. The present series of investigations were, therefore, planned to survey the free-living population of Indian grasshoppers cytologically with a view to finding structural changes in the chromosomes and, if they are present, to determine their nature, extent and behaviour.

We collected grasshoppers for our investigations at Kashba, a locality in the southern suburbs of Calcutta, during the months of June to August, 1947, as the place is rich in grasshopper fauna and a number of species belonging to different genera could be collected and examined. During the course of our work in June 1947, we detected the presence of a supernumerary chromosome in two grasshoppers belonging to an undetermined species of the genus *Aiolopus*,* out of a total of 26 specimens. In this paper we shall designate the species as *Aiolopus* sp. 'B' in order to distinguish it from another congeneric species found in the same locality which has been termed *Aiolopus* sp. 'A'. In addition to their normal chromosome complement, each of the two individuals had an additional chromosome of very small size in all the dividing cells of their testes. The present report

* A specific determination was unfortunately not possible (Uvarov, personal communication). Several examples of *Aiolopus* sp. 'A' and 'B' are now kept in the British Museum (Natural History) and in the collections of Zoological Survey of India, Calcutta.

deals with the cytology of these two individuals. The size and behaviour of the extra chromosome in both of them are exactly similar and, therefore, the description applies to both of them. It is also necessary to include in this paper a brief account of the structure and behaviour of the chromosomes in the testes of normal individuals because this genus has been studied for the first time.

Chromosomes of normal individuals were studied mainly from sectioned material. For the routine survey work, smearing of the cells of the testes was made on two slides from each individual. In both cases, the material was fixed in Belling's fluid and stained in Feulgen's stain.

Observation

Chromosomes in normal males.—The normal diploid complement of *Aiolopus* sp. 'B' consists of 23 acrocentric chromosomes which are roughly classified in the spermatogonial metaphase plate into three size classes *viz.*, (i) three pairs of small dot-shaped chromosomes, (ii) seven long and (iii) five pairs of medium-sized chromosomes (Fig. 1). The sex chromosome is distinguishable at the early spermatogonial divisions by its negative heteropycnotic behaviour and is one of the longest chromosome in the complement.

Table I gives the mean lengths of the chromosomes calculated from the measurements obtained from 20 carefully selected second division metaphase plates where the chromosomes are evenly placed without any bending or fore-shortening.

Table I

Mean lengths of the second division metaphase chromosomes in Aiolopus *sp B.*

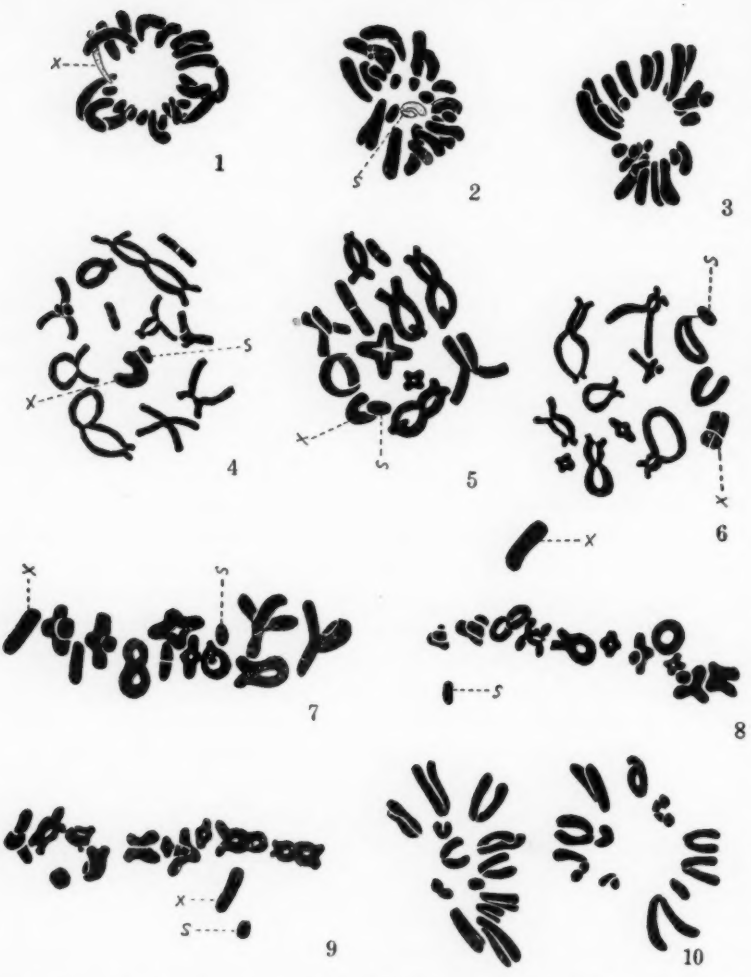
Chromosome type	Mean length in micra
Long ..	5·07 4·58 3·88
Medium ..	3·35 3·08 2·89 2·74 2·54
Short ..	1·54 1·42 1·10
X ..	4·24

Meiosis is normal and orthodox. The mean chiasma frequency per nucleus, calculated from a study of 25 early diplotene nuclei, is 18·04. The total number chiasmata per nucleus varies between 15 and 23 at diplotene, between 13 and 20 at diakinesis and between 12 and 19 at metaphase.

Behaviour of the supernumerary chromosome.—Spermatogonial metaphase stages were very rare in the limited number of slides prepared for the study. Moreover, smear preparations are not suitable for a study of the top view of spermatogonial metaphase chromosomes. Fortunately, a few clear plates were obtained which invariably showed 24 chromosomes (Figs. 2 and 3), instead of usual 23 found in the normal males. Fig. 2 illustrates one such plate where the supernumerary chromosome shows a slight negative heteropycnosis and the daughter chromatids are separated distally, showing a clear split between them. The sex chromosome, on the other hand, is stained like the autosomes and therefore, indistinguishable in the complement.

The supernumerary first becomes identifiable clearly in the meiotic cells at the diplotene stage, when all the normal autosomes have paired and formed bivalents, but the supernumerary and the X lie as univalents. In order to determine the homology of the former, we carefully looked for its position in relation to the normal chromosomes. Chiasma pairing between the additional and normal chromosomes, was never observed but the former has been frequently found to be associated with the X (45 times out of a total of 80 diplotene and diakinesis nuclei examined). Fig. 4 illustrates a diplotene nucleus where the fragment is connected with the X by a thin thread-like structure. Fig. 5 is drawn from a diakinesis nucleus in which the fragment is closely associated with one end of the X. That the supernumerary may lie within the nucleus quite independently of the X is illustrated in Fig. 6, which represents a diplotene nucleus where the fragment is independent of the X and connected with an autosome.

In spite of the earlier association of the supernumerary in a certain percentage of cases with the X, they behave quite independently of each other during the formation of the metaphase spindle. Like the X chromosome the supernumerary sometimes forms an accessory plate on one side of the spindle and at other times one or both of them may arrange themselves on the equatorial plane. Figs. 7-9 illustrate some of the metaphase arrangements of the supernumerary and the X chromosome. A count was made to determine the relative frequencies of the following two types of metaphase spindle: (i) cases where the X and the supernumerary lie on the same side of the spindle, and (ii) those in which the X lies on one side and the super-



FIGS. 1–10. Fig. 1. Early spermatogonial metaphase of normal individual, × 1750. Fig. 2. Spermatogonial metaphase of supernumerary individual × 1750. Fig. 3. Spermatogonial metaphase of supernumerary individual where the supernumerary is not distinguishable, × 1750. Fig. 4. Diplotene stage, supernumerary connected with the X by a thin thread-like structure, × 1750. Fig. 5. Diakinesis stage, supernumerary closely associated with the sex chromosome, × 1750. Fig. 6. Diplotene stage, supernumerary connected with an autosome, × 1750. Fig. 7. Metaphase I, X and supernumerary on the equatorial plate, × 1750. Fig. 8. Metaphase I, X and supernumerary polarized in opposite poles, × 1750. Fig. 9. Metaphase I, X and supernumerary polarized towards the same pole, × 1750. Fig. 10. Anaphase I, X and supernumerary passing to the same pole, × 1750.

numerary on the other, and it was found that both types are about equally frequent.

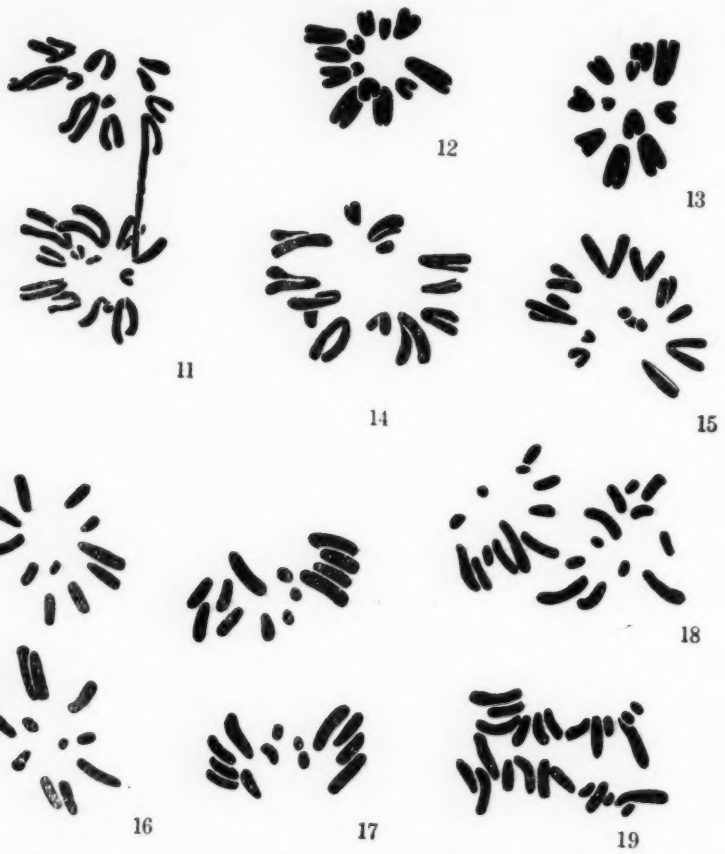
In order to make an estimate of the size of the supernumerary chromosome, its measurements were made from those nuclei at the metaphase stage of the first meiotic division where they form accessory plates like the X. Fifteen such nuclei were examined for the purpose in each of the two testes. The measurements varied beween 1·3–1·8 micra, the mean being 1·6 micra in both the testes. It is possible to compare the sizes of the supernumeraries found in different individuals under different conditions of fixation and spiralization by calculating the ratio between the mean lengths of the X and the supernumerary in any one individual, provided measurements are taken from nuclei in the same stage of the division and the slides are similarly treated during preparation. The mean length of the X differs a little in the two individuals, being 4·4 and 4·3 micra respectively. The X is, therefore, 2·7 times longer than the supernumerary.

During anaphase of the first division, the supernumerary may either go to the same pole along with the X (Fig. 10), or the X and the supernumerary pass to the opposite poles (Fig. 11). As a result of these two types of segregation, there are four types of second division metaphase plates: (*a*) with 11 autosomes plus the supernumerary and the X (Fig. 12), (*b*) with 11 autosomes (Fig. 13), (*c*) with 11 autosomes and the X (Fig. 14) and (*d*) with 11 autosomes and the supernumerary (Fig. 15). The frequency of the four kinds is nearly equal.

The X and supernumerary divide equationally at the second meiotic division. Fig. 16 is a polar view of the second division anaphase showing 11 autosomes, while Fig. 17 is a side view of the same stage showing 13 chromosomes. There are two other types of second division anaphase figures which contain 12 chromosomes, *viz.*, (i) 11 autosomes plus the supernumerary (Fig. 18) and (ii) 11 autosomes plus the X (Fig. 19).

Discussion

The genetical consequences of the production of four types of sperms as a result of independent segregation of the X and the supernumerary reported above are obvious. In addition to the production of normal individuals, both males and females, would be produced with a supernumerary chromosome. Further, crossing between the latter is likely to produce individuals with two, or, in the later generations more than two supernumeraries. The number of specimens examined by us is too small for detecting them even if they occur in the population.



FIGS. 11–19. Fig. 11. Anaphase I, X and supernumerary passing to the opposite poles, × 1750. Fig. 12. Metaphase II, eleven autosomes plus the supernumerary and the sex chromosome, × 1750. Fig. 13. Metaphase II, eleven autosomes only, × 1750. Fig. 14. Metaphase II, eleven autosomes plus the sex chromosome, × 1750. Fig. 15. Metaphase II, eleven autosomes plus the supernumerary, × 1750. Fig. 16. Anaphase II, with eleven autosomes, × 1750. Fig. 17. Anaphase II, with thirteen chromosomes, × 1750. Fig. 18. Anaphase II, eleven autosomes plus the supernumerary, × 1750. Fig. 19. Anaphase II, eleven autosome plus the sex chromosome, × 1750.

Short title : *Supernumerary chromosome in grasshopper.*

The identical size of the supernumerary and its behaviour in the two individuals leave no doubt as to their genetic relationship. A discussion, on the origin and nature of the extra chromosome found by us, cannot be attempted without more extended observations and, therefore, must be reserved for a future publication. The study is being continued and in the

meantime we have thought it expedient to record our observations to draw attention of the cytologists for collaboration in securing data with regard to the geographical distribution and the frequency of supernumerary individuals in the population without which, as pointed out by White (1945), it will be impossible to tell what role they play in the population dynamics of species in which they occur.

Summary

1. Two individuals out of a total of 26 specimens of *Aiolopus* sp. 'B' examined cytologically showed in all the dividing cells of their testes a super-numerary chromosome of about one-third of the size of the X.

2. The normal diploid number in the species is 23 and it has an XX-XO mechanism of sex-determination.

3. The additional chromosome never forms a chiasma with any member of the normal chromosome set, but it has been found, in about half of the diplotene and diakinesis nuclei, to be associated with the X chromosome.

4. The supernumerary chromosome segregates without division independently of the X in the first meiotic division and divides equationally at the second meiotic division. As a consequence of this behaviour, it is regularly included in half of the sperms produced.

Acknowledgment

We are indebted to Dr. B. P. Uvarov, F.R.S., British Museum (Natural History), for the identification of the material.

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ADDITIONS TO FUNGI OF MADRAS—X

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Received October 18, 1950

Phytophthora cinnamoni Rand.

Tucker, G. M., "Taxonomy of the genus *Phytophthora* de Bary," *Res. Bull. Coll. Agri. Univ., Mossouri*, 1931, No. 153, 183.

On stem and roots of *Cinchona ledgeriana* Moens, *Chinchona succirubra* Pav. and other species, Govt. Cinchona Plantations, Anamalais, 15-10-1949, T. S. Ramakrishnan.

Large-scale infection was noticed in the plantation. The fungus was found to cause canker on the stem, collar rot and root rot, finally resulting in the drying of branches or death of plants. The incidence became pronounced after the south-west monsoon.

Empusa aphidis Hoffman.

Cotton, A. D., *Trans. Brit. Myc. Soc.*, 1918–20, **6**, 201.

On aphids (*Macrosiphum solanifolii*?) infesting leaves of *Fagopyrum esculentum* Moench. (Ploygonaceæ), Nanjanad (Nilgiris), 14–8–1950, T. S. Ramakrishnan.

Large numbers of insects had been killed and were sticking to the leaves. The fungus had enveloped many of them forming creamy white growths. Stout hyphæ were also present inside the bodies of the insects and extended into the legs. Conidiophores were unbranched and closely packed. Conidia were hyaline or subhyaline, vacuolate, oval or elliptical and thin-walled. A papillate protrusion was present at the base. Germination was by the formation of a germ tube.

Leveillula taurica (Lev.) Arn.

Salmon, E. S., *Ann. Bot.*, 1906, **20**, 187–200.

On living leaves of *Passiflora calcarata* Mast. (Passifloraceæ), Botanic Gardens, Ootacamund, 15–8–1950, T. S. Ramakrishnan.

Definite spots are lacking but on the upper surface light green areas are evident. On the corresponding lower surface white powdery, vein-limited patches made up of the conidiophores and the conidia are present.

Nectria diploa Berk. and Curt.

Petch, T., *Trans. Brit. Myc. Soc.*, 1921, **7**, 157.

On scale insects infesting stem and twigs of *Artocarpus integrifolia* L. (Moraceæ), Agricultural Research Station, Pattambi (Malabar), 15–8–1950, N. V. Sundaram.

A severe infection of the scale insects infesting the twigs was prevalent. Most of them were covered by whitish or cream-coloured growths with oblique or lateral sporodochia. Numerous hyaline, arcuate, 3–5 septate conidia were present. Arranged all round or sometimes directly on the whitish growths were clusters of bright red perithecia. The asci were eight-spored with 1-septate, light coloured, elliptic ascospores.

Sphærostilbe auranticola (Berk. and Broome) Petch.

Petch, T., *Trans. Brit. Myc. Soc.*, 1921, **7**, 158.

On *Mytilaspis beckii* New., infesting *Citrus reticulata*, Wynad (Malabar), 11–8–1950; Singampati (Tinnevelly), 15–10–1949, M. S. Subbiah.

This scale is known to infest orange, pepper and various other hosts. The characteristic club-shaped orange red synnemata of the conidial phase were present in large numbers on the scales.

Achorella vaccinii sp. nov.

Stromata cæspitose, globose, black, hypophyllous, with one or more loculi, subepidermal, erumpent, up to 1 mm. in diam.; asci cylindric to cylindric clavate, stipitate, curved or straight, wall hyaline, 90–128 × 11–13 μ, 8-spored, paraphysate, paraphyses septate and branched; ascospores 2-celled, monostichous, olive brown, elliptic or oblong, cells unequal, often constricted at the septum, 11–15 × 5–7 μ.

Stromata cæspitosa, globosa, nigra, hypophylla, uni vel pluriloculata, subepidermalia, erumpentia, usque 1 mm. diam.; asci cylindrici vel cylindrico-clavati, stipitati, recti vel curvati, parietibus hyalinis, ornati, 90–128 × 11·13 μ, octospori, paraphysati, paraphysibus septatis et ramosis; ascosporidia bicellata, monostichia, olivaceo-brunnea colore, elliptica vel oblonga, cellulis inæqualibus, constricta ad septum, 11–15 × 5–7 μ.

On living leaves of *Vaccinium leschnaultii* W. (Vacciniaceæ), Kodaikanal, 25–12–1949, K. V. Srinivasan.

The stromata occur in groups on the lower surface appearing as clusters of rounded black bodies easily thumbed off. Each group has a foundation of narrow or broad internal dark pseudoparenchymatous mass occupying

one or two layers of the mesophyll and breaking through the epidermis. The ascospores are hyaline when young and olive brown when mature. The two cells of the ascospore are usually unequal. The upper cell is slightly broader and more or less conical in shape. The loculi may be one or more in each lobe of the cluster and measure 130–70 × 100–10 μ.

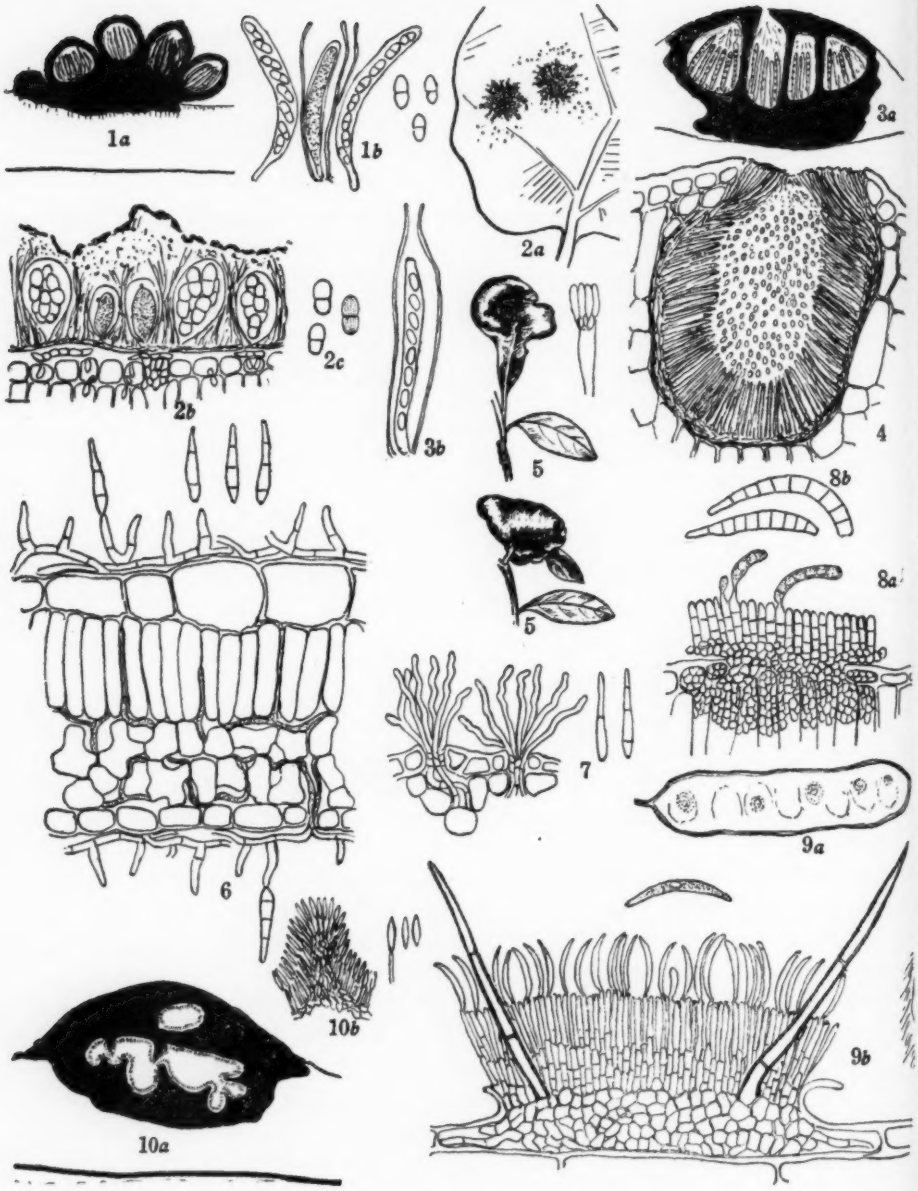
Hysterostoma brevilobi sp. nov.

Superficial hyphæ none; ascomata in circular groups, crowded, epiphyllous, black, globose; internal hyphæ subcuticular and in the upper two layers of cells of the mesophyll; asci obovate, wall thickened at the apex in young asci, hyaline, 73 × 51 μ (52–89 × 41–68), 8-spored, paraphysate, paraphyses hyaline, gelatinising; ascospores hyaline when young but dark brown later, 1-septate, finely verrucose, oblong, constricted at the septum, conglobate, 37 × 21 μ (20–42 × 13–24).

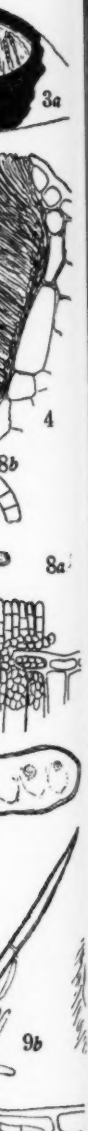
Mycelium superficiale nullum; ascomata circinnatim disposita, gregaria, epiphylla, nigra, globosa; hyphæ interiores subcuticulares et intracellulares; asci obovati, ad apicem incrassati, hyalini, 73 × 51 μ (52–89 × 41–68), octospori, paraphysibus hyalinis demum gelatinosis; ascosporidia primo hyalina dein atrobrunnea, 1-septata, minute verruculosa, oblonga, ad septum constricta, conglobata, 37 × 21 μ (20–42 × 13–24).

On living leaves of *Jasminum brevilobum* A.DC. (Oleaceæ), Kodaikanal, 24-12-1949, K. V. Srinivasan.

Circular black groups of densely arranged ascomata are formed on the upper surface of the leaves without any definite spots. The infected portion is pale green in colour. Superficial hyphæ are absent but the ascomata rise close together in apparently radiating lines. Ascomata have one or more ill-defined loculi. The opening is hysteriaceous. The asci are mixed with gelatinising paraphyses which give a disorganised appearance to the context round the asci. The hypostroma consists of groups of dark brown cells spreading subcuticularly. From these other hyaline hyphæ penetrate into the epidermal and subepidermal cells. In the stomatal openings larger groups of the dark cells are present. In the young asci the wall in the upper half and the apex is much thickened and glistening. But in older asci it is thin. The ascospores are hyaline when young but gradually become dark brown and are massed together inside the ascus. On old spores fine projections are observed arranged in longitudinal striæ. *Ferrarisia jasmini* Doidge has been recorded on *Jasminum* sp. from S. Africa (Doidge, 1942). But the fungus under study has no superficial hyphæ nor are the peculiar intracellular hyphæ present.



FIGS. 1–10



FIGS. 1–10. Fig. 1. (*a*) Stromata of *Achorella vaccinii* (semidiagrammatic); (*b*) asci paraphyses and ascospores, × 250. Fig. 2. (*a*) Portion of leaf of *Jasminum brevilobum* showing the ascomata of *Hysterostoma brevilobi*; (*b*) Section through one ascoma; and (*c*) ascospores, × 250. Fig. 3. (*a*) Section through a stroma of *Phyllachora rhamni* (diagrammatic); (*b*) ascus and paraphyses. Fig. 4. Section of pycnium of *Xenostele indica*, × 300. Fig. 5. Leaves of *Azalea indica* affected by *Exobasidium vaccinii* (½ size) and a basidium with basidiospores, × 250. Fig. 6. Section of leaf with *Cercospora pilosa*, × 250. Fig. 7. Section of leaf with *Cercospora vestita* and conidia, × 250. Fig 8. (*a*) Section through an acervulus of *Scolecosporium phœbii*; (*b*) conidia, × 250. Fig. 9. (*a*) Pod of *Albizzia lebbeck* with acervuli (½ size); (*b*) section of an acervulus, × 250. Fig. 10. (*a*) Section of a stroma of *Sclerotiopsis indica* (diagrammatic); (*b*) conidiophores and conidia, × 250.

Phyllachora rhamni sp. nov.

Spots amphigenous, circular, 1·5–2 mm. wide, indefinite, yellow, isolated or confluent; stromata one or two in each spot, amphigenous raised, black, shining, clypeate, with one or more loculi, loculi 170–240 μ high and 150–290 μ broad, ostiolate; asci cylindric, hyaline, thin-walled, 8-spored, 106–128 × 8–11 μ, paraphysate, paraphyses filiform; ascospores uniseriate, oval, hyaline, 1-celled, 10–14 × 5·7 μ.

Maculæ amphigenæ, orbiculares, 1·5–2 mm. lat. indefinitæ, flavidæ, isolatæ vel confluentes, stromata solitaria vel duo in singulis maculis, amphigena, elevata, atra, micantia, clypeata, uni vel pluri loculata, loculis 170–240 μ alt. et 150–290 μ lat., ostiolata; asci cylindrici, hyalini, tenuiter tunicati, octospori, 106–28 × 8–11 μ, paraphysati, paraphysibus filiformibus; ascosporidia uniseriata, ovata, hyalina, unicellata, 10–14 × 5–7 μ.

On living leaves of *Rhamnus wightii* W. and A. (Rhamnaceæ), Ootacamund, 15–8–1950, T. S. Ramakrishnan.

The spots have no definite margin but are yellow and circular. These are isolated or sometimes two or three coalesce. One shining black stroma is usually seen in each spot. The stroma is more elevated on the upper surface and extends throughout the thickness of the leaf. One to four loculi have been observed in each stroma. Paraphyses are numerous and are filiform and slightly longer than the asci.

Xenostele indica Thirumalachar.

Ramakrishnan, T. S. and K., *Proc. Ind. Acad. Sci.* B, 1947, **25**, 28.

Thirumalachar, M. J., *Curr. Sci.*, 1948, **17**, 26.

The telial stage alone of this rust had been observed so far. Recently a severe infection of young leaves of *Neolitsea zeylanica* was observed. When the young infection spots were examined pycnia were found by the side of

young telia. These were oval, subepidermal and deep seated, mostly epiphyllous and rarely hypophyllous. The ostiole was more or less on a level with the epidermis without any filaments projecting beyond the opening. They measured 150–230 μ deep and 110–70 μ broad. They were yellow to orange in colour with a pronounced cavity in the middle.

Exobasidium vaccinii (Fuck.) Wor.

Hotson, J. W., *Phytopathology*, 1927, **17**, 207.

On living leaves of *Azalea indica* (Ericaceæ) causing the development of galls, Ootacamund, 15–7–1950, T. S. Ramakrishnan.

Azalea indica is an ornamental shrub cultivated in many gardens on the Nilgiris. During the month of August some of the plants in the Botanic Gardens were infected by *Exobasidium*. Entire leaves or portions of leaves developed into light green thick laddle-shaped galls. These galls were one to three centimetres long and about half a centimetre thick. They were flat or more often involute or doubled with the upper surface concave. At the tips of branches several consecutive leaves were hypertrophied. The galls were covered by a white powdery growth. Some of the galls especially old ones turned into varying shades of pink and later rotted into brown masses.

The basidia were amphigenous in tufts, clavate, hyaline and non-septate, with 4–5 sterigmata. The basidiospores were more or less cylindrical 18–20 $\times$ 3 μ, slightly narrowed at the base and erect or rarely curved. Septation was not observed. They germinated readily and produced acicular secondary spores. This is the first record of the fungus on this host from India.

Cercospora pilosa sp. nov.

Spots indefinite, mycelium internal and superficial, internal hyphæ hyaline, external hyphæ light brown, amphigenous, but more on the lower surface, effuse growth; conidiophores simple or branched, borne on external hyphæ, erect, subhyaline to brown, 1–3 septate, 24–70 $\times$ 5–7 μ, not fascicled; conidia obclavate, hyaline or light brown, 1–4 septate, 45 $\times$ 5 μ (26–78 $\times$ 5–7 μ).

Maculæ indefinitæ, mycelia interna et superficialia, hyphis internis hyalinis, hyphis superficialibus levibrunneis, amphigena, sæpius hypophylla, effusa; conidiophori simplices vel ramosi, hyphis superficialibus insidentes, ascendentes, subhyalini vel brunnei, 1–3 septati, 24–70 $\times$ 5–7 μ,

nonfasciculati; conidia obclavata, hyalina vel levi brunnea, 1–4 septata, 45 × 5 μ (26–78 × 5–7).

On living leaves of *Triumfetta pilosa* Roth. (Tiliaceæ), Kodaikanal, 24-12-1949, K. V. Srinivasan.

The presence of infectoin is located by the tawny effuse growths more or less roundish in outline prominently visible on the lower surface of the leaves. The conidiophores are formed outside the tissues on the external mycelium which lies close to the surface of the leaves. These are developed all along the length. The parent hyphæ are prostrate while the conidiophores project out. The conidium easily separates from the stalk and has a prominent scar at the base, appearing as a dark line. The fungus under study comes near *Cercospora triumfettæ* Syd., but differs from it in the fewer septa and significantly smaller size of the conidia.

Cercospora vestita sp. nov.

Spots indefinite, prominent hypophyllously, greenish black; conidiophores fascicled, hypophyllous, flexuous, olive brown, non-septate, 50–75 × 4–5 μ, stroma absent; conidia hyaline to subhyaline, 1–4 septate, straight or curved, cylindric to obclavate, 34–60 × 2–4 μ.

Maculæ indeterminatæ, prominenter hypophylla, nigroviridia; conidiophori fasciculati, hypophylli, flexuosi, olivaceo-brunnei, non-septati, stromate nullo, 50–75 × 4–5 μ; conidia hyalina vel subhyalina, 1–4 septata, recta vel curvata, cylindrica vel obclavata, 34–60 × 2–4 μ.

On living leaves of *Leucas vestita* Benth. (Labiatæ), Kodaikanal, 24-12-1949, K. V. Srinivasan.

On old leaves the infected portions present a greenish black dotted appearance on the lower surface. But when fresh, a whitish powdery formation of conidia can also be seen on the spot. The conidiophores are formed in tufts emerging out through most of the stomata in the infected region. Stromatic development at the base of the clump is not discernible. The conidiophores are highly flexuous throughout the length and geniculate at the apices. Septation is not distinct. Conidia are hyaline at first. Some of them become subhyaline with age.

Heterosporium echinulatum (Berk.) Cooke.

Burt, C. C., *Trans. Brit. Myc. Soc.*, 1936, **20**, 207–15.

On living leaves of *Dianthus caryophyllus* L. (Caryophyllaceæ), Ootacamund, 16-8-1950, T. S. Ramakrishnan.

This fungus was found to cause depressed oval or elliptic greyish white spots on the leaves and shoots of one variety of the cultivated pink in the Botanic Gardens. The characteristic conidiophores and conidia were present on the spots. The perfect stage of the fungus *Didymellina dianthi* Burt was not observed.

Scolecosporium phœbei sp. nov.

Acervuli minute, erumpent, effuse, black, scattered, epiphyllous; conidiophores coloured, compactly arranged, septate; conidia terminal, light to dark brown, arcuate, 8–10 septate, 42–85 × 8–11 μ tapering towards the apex.

Acervuli minuti, erumpentes, effusi, atri, sparsi, epiphylli; conidiophori, colorati, compacte dispositi, septati; conidia acrogena, levi vel fusco-brunnea, arcuata, 8–10 septata, 42–85 × 8–11 μ, apicata tenuissima.

On living leaves of *Phœbe paniculata* Nees (Laurineæ), Ootacamund, 16–8–1950, T. S. Ramakrishnan.

The acervuli project out as minute dark raised structures on the upper surface. The stroma is multicellular and mostly occupying the palisade tissue in the region. After bursting through the epidermis the stromatic tissue curves out on the surface. From the upper layers a close set palisade of conidiophores projects. The conidiophores are reddish brown at the base becoming lighter towards the apex. The conidia are light coloured when young and become darker with age. The terminal cell of the conidium is longer than the rest and narrow resembling a rostrum. The basal cell is flattened. On the basis of these characters the fungus has been placed in this genus.

Colletotrichum compactum sp. nov.

Spots circular, acervuli numerous, arranged in concentric lines, black, of different sizes, intraepidermal later erumpent; setæ few, brown, septate, unbranched, 95–125 × 7–9 μ; conidia fusoid, arcuate, 27 × 4 μ (20–38 × 3–5), hyaline, one-celled, conidiophores subhyaline and compactly arranged.

Maculæ orbiculares; acervuli plures, circinnate dispositi, atri, magnitudinis variæ, epidermales, late erumpentes; setulis sparsis brunneis, septatis, simplicibus, 95–125 × 7–9 μ; conidia fusoidea, arcuata, 27 × 4 μ (20–38 × 3–5) hyalina, unicellata, basidia subhyalina conglutinata.

On living pods of *Albizzia lebbeck*, Benth. (Mimosoideæ) Coimbatore, 10–12–1949, T. S. Ramakrishnan.

Roundish depressed spots develop on the pods. The affected fruits shrivel up and are shed prematurely. Numerous small black acervuli are formed in each spot and are arranged in concentric rings. These originate intra-epidermally and burst through the epidermis at a late stage. Few setæ are formed in each acervulus. The conidiophores are subhyaline and compactly arranged. The spores are hyaline falcate and guttulate or with granular contents. The spores often remain in a mass bound together in mucilage and do not easily separate when fresh. When healthy pods are inoculated with the fungus infection symptoms are evident in five to six days.

Colletotrichum lebbeck (Syd.) Petrak (Petrak, 1927) has been recorded on fruits of this host from the Philippines. But the conidia are oblong and measure only 12–18 × 3·5–4·5 μ. The fungus under study is obviously different.

Septoria polygonicola (Lasch) Sacc.

Saccardo, P. A., *Syll. Fung.*, 1892, **3**, 380.

On living leaves of *Fagopyrum esculentum* (Polygonaceæ), Ootacamund, 16–8–1950, T. S. Ramakrishnan.

Several minute, ochraceous to pink spots are formed on the leaves. In the middle of each spot depressions are present on the upper surface indicating the location of pycnidia. These are deeply sunk and produce numerous filiform hyaline straight or curved or flexuous spores measuring 50–60 × 1–1·5 μ. *Depazea polygonicola* Lasch, had been recorded on this host from Europe. This has been made synonymous with *Septoria polygonicola* (Lasch) Sacc. The fungus under study closely resembles the latter and is identified as such.

Sclerotiopsis indica Ramakrishnan and Srinivasan sp. nov.

Stromata epiphyllous, raised, conical or hemispherical, black, shining 1–2 mm. in diameter, deep-seated, context parenchymatous with labyrinthine loculi, ostiole not present; conidia fusoid, hyaline, 1-celled, 17–22 × 2–4 μ, borne on crowded unbranched stalks.

Stromata epiphylla, elevata, conica vel hemispherica, nigra, micantia, 1–2 mm. in diam., immersa, textura parenchymatica, loculis labyrinthiformibus, absque ostiolo; conidia fusoidea, hyalina, continua, 17–22 × 2–4 μ, basidiis simplicibus, dense stipatis, insidentia.

On living leaves of *Ilex wightiana* Wall. (Aquifoliaceæ), Kodaikanal, 24–12–1949, K. V. Srinivasan.

The stromata are scattered over the upper surface as black shining convex or conical growths. They are covered by the epidermis. No definite spot is evident but surrounding the stroma is a narrow raised ridge. The position of the stroma is indicated on the lower surface by a slightly raised discoloured patch.

The sporebearing cavities are very much lobed and extend in different directions. Lining the cavities throughout their extent are closely arranged hyaline conidiophores. Each one is unbranched and bears at its tip a spindle-shaped hyaline spore. The context of the stroma is made up of olive brown pseudoparenchymatous cells. Mature stromata exhibit splitting along the periphery.

I am indebted to the Government Entomologist, Coimbatore, for the identification of the insects and to Rev. Dr. H. Santapau of St. Xavier's College, Bombay, for the Latin translations.

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NITRIFICATION IN RELATION TO CATION ABSORPTION BY PLANTS

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The question of how plants obtain their mineral constituents from the soil is still engaging the attention of both the plant physiologists and soil chemists. The view put forward by Whitney (1892) and later on Cameron that the source of plant nutrients is the soil solution was generally accepted until recently when Jenny and Overstreet (1939) brought forth evidence that the cations migrate by a direct exchange phenomena between roots and soil colloids. However, as pointed out by Hoagland (1944) and Burd (1947), certain ions particularly nitrate ions are not adsorbed to any important degree by the soil colloid and anions present in the soil solution must necessarily be accompanied by equivalent cations. Even accepting the possibility of contact exchange, it could not result according to Burd (*loc. cit.*) in the accumulation of metallic cations by the plant, as the characteristic chemical complexes of plant root surfaces are largely carbohydrates (celluloses and hemicelluloses and though some such compounds have acidoid properties, they are so ' weak ' (in the chemical sense) that it is difficult to see why H^+ should leave them for clay particles which appear to be in general strong acids. This does not seem to have taken into account the possible influence of proteins and pectins, which may part with their H^+ ions.

If the soil solution is the source of plant nutrients, there must be some solvent other than water for making available the plant nutrients from the solid phase. Even in this regard, there is not complete unanimity of opinion regarding the possible solvents. While the carbon dioxide evolved in the soil due to microbial activity or excreted by roots of plants, can be a possible solvent, it cannot by itself account for the availability of soil constituents to the plants (Hoagland, *et al.*, 1916, 1918, 1930; Thomas, 1930; Oberholzer, 1936). Hibbard (1919) pointed out that the changes in H-ion concentration, by the change of organic nitrogen, phosphorus and sulphur to their mineral acids are probably adequate to account for the increased solubility of calcium, magnesium and potassium.

Stephenson (1936) expressed the view that the strong nitric acid formed during the process of nitrification may be largely responsible for the availability of cations to the plant. From a calculation of the soil reaction which

would be induced by nitric acid equivalent to nitrogen in the 920 bushel crop of wheat, Davidson (1944) comes to the conclusion that nitric acid formed in the soil in the process of nitrification was the cation solvent, looked for by scientists. In view of the difference of opinion in the literature, an attempt has been made in this paper to examine the importance of soil nitrification process as regards solvent action and as annion carrier of cations to the plant.

Experimental

Laboratory studies of nitrification of different manures and fertilisers and its effect on the solubility of cations

Five hundred gm. of soil lots were given the following twelve treatments in duplicate at the rate of 30 mgm. per hundred gm. of soil:— (1) No manure, (2) F.Y.M., (3) Compost, (4) Green manure cowpeas, (5) Activated sludge, (6) Mustard cake, (7) F.Y.M.+ P_2O_5+ K_2O, (8) Activated sludge + P_2O_5+ K_2O, (9) Mustard cake + P_2O_5+ K_2O, (10) Ammonium sulphate + P_2O_5+ K_2O, (11) Compost + Ammonium sulphate + P_2O_5, (12) F.Y.M. + Ammonium sulphate + P_2O_5. In treatments seven to ten phosphate and potash were added at the rate of 30 mgm. P_2O_5 and K_2O each, to study their effect on nitrification and on the solubility of cations. In treatments 11 and 12, nitrogen has been added half the amount in the organic form and the other half as ammonium sulphate. They were incubated in the laboratory at 32° C. and at 12% moisture level ($\frac{1}{3}$ saturation capacity which was found to be optimum. The moisture was made up every three days. The loss of water was not more than 3 c.c. in a week.

Nitrate nitrogen was determined periodically every fortnight by phenol-disulphonic acid method and at the end of the experiment soils were analysed for calcium, magnesium and potassium in the 1:5 soil: water extract. Calcium was precipitated as oxalate and estimated volumetrically with permanganate while magnesium was estimated in the filtrate gravimetrically by weighing as magnesium pyrophosphate. Potassium was estimated by cobaltinitrate method. Carbon was estimated both initially and finally by chromic acid wet oxidation method to estimate the amount of carbon dioxide evolved during the process.

The following table gives the amount of nitrate produced and the cations made soluble and the total carbon oxidised during a period of three months incubation.

It is readily seen from the table that in the first six treatments where no phosphate and potash were added, the amount of water-soluble cations

TABLE I

Nitrification in relation to availability of cations

(1 : 5 soil : water solubles per 100 gm of soil)

Treatment	NO_3N		Ca		Mg		K		C lost in mg. per 100 g. soil	Sum of cations	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*		*a*	*b*
1 No manure ..	3·5	0·25	5·1	0·21	2·5	0·21	Traces		44	7·6	0·47
2 F.Y.M. ..	4·8	0·34	8·3	0·42	7·3	0·61	1·10	0·03	261	16·7	1·06
3 Compost ..	7·8	0·56	12·6	0·63	4·6	0·38	3·3	0·09	168	20·5	1·10
4 G. M. Cowpeas ..	12·4	0·89	18·3	0·92	7·5	0·63	3·6	0·09	142	29·4	1·64
5 Act. sludge ..	13·7	0·98	22·6	1·13	12·1	1·01	Traces		152	34·7	2·14
6 Mustard Cake ..	14·7	1·05	27·8	1·39	10·60	0·88	,,		166	38·4	2·27
7 '2' + P_2O_5 K_2O ..	4·1	0·29	28·5	1·43	12·6	1·05	4·6	0·12	211	45·5	2·60
8 '5' + P_2O_5 K_2O_4 ..	13·5	0·95	39·8	1·99	11·9	0·99	5·5	0·14	167	57·2	3·13
9 '6' + P_2O_5 K_2O ..	16·8	1·20	41·3	2·07	11·6	0·97	7·1	0·18	141	60·0	3·22
10 Amm. sulp. P_2O_5 + K_2O ..	23·5	1·68	31·0	1·55	6·80	0·57	4·3	1·10	71	42·1	2·23
11 Compost + S/A + P_2O_5 ..	17·7	1·25	42·9	2·15	11·9	0·99	..	..	126	..	..
12 F.Y.M. + S/A + P_2O_5 ..	15·0	1·07	38·6	1·93	11·5	0·96	..	..	116	..	..

(*a*) represents mgm. per 100 gm. of soil (1 : 5 water extract) ;
(*b*) represents milligram equivalents per 100 gm. of soil (1 : 5 water extract).

in terms of equivalents increase with the amount of nitrate formed and practically bear a simple ratio of 2 : 1. Thus the water-soluble cations bear a simple and direct relationship to the nitrate. The initial soil contained only ·5 mg. of nitrate nitrogen, 2·8 mg. of calcium, 1·8 mg. of magnesium and traces of potassium per 100 gm. of soil (1 : 5 water solubles).

In the treatments where artificial phosphate and potash were added, the amount of water-soluble cations present in the soil were more than the corresponding figures for organic manures because of the extra amount of phosphate added in the form of inorganics and there is the clear indication, that nitrification has resulted in the increased solubility of cations proportional to the amount of nitrate formed. In the case of the treatment ammonium sulphate with phosphate and potash, the amount of cations made soluble appear to be less compared to other similar treatments, probably because of the difficulty in solubilising the soil cations compared to easily available cations from the organic matter of other organic manures. In these experiments inorganic phosphate and potash have been added to the manures in treatments seven to twelve at the rate of 30 mgm. of P_2O_5 and K_2O each per 100 gm. of soil.

Carbon oxidised during this period is also given in the table for each treatment. The amounts oxidised varied from 44 mgm. of carbon in the case of no manure to 261 mgm., in the case of the treatment with F.Y.M.

There appears to be no correlation between carbon oxidised and cations made soluble. Carbon dioxide appears to play a very insignificant part and nitrification may largely explain the increased availability of cations to the plant.

Relationship between nitrate and cation absorption by plants

Although ammonium ion can also act as the carrier of nitrogen, it is mostly in the form of nitrate that nitrogen is absorbed from most of the ordinary soils (Nightingale, 1937; Russel, 1937; and Hoagland, 1944). If nitrate is assumed to be the carrier of nitrogen, it must be accompanied by an equivalent amount of cations to ensure electrical neutrality. Compared to the other anions like PO_4 and SO_4, nitrogen forms the bulk of the anions that enter the plant. Thus the plant of high nitrogen content must be high in cations also if nitrogen is absorbed in the form of nitrate and nitrate is the carrier of cations.

Parker and Troug (1920) found the relationship between high nitrogen and high calcium although they were studying from a different point of view. Stephenson (1936) however showed that nitrogen (taken as nitrate ion) accounts for more than the cations in a number of plants but his results are vitiated by the fact that he had considered only parts of the plants which might have specialised functions requiring a differential accumulation, thus masking the real proportion of cations and anions absorbed.

The data available in literature in the chemical composition of plants, has been examined to find out the nature of relationship existing between nitrogen and cations taking the plant as a whole. In Table II below are presented compositions of different plants, namely N, K, Mg, Na and Ca and are expressed in equivalents per thousand gm. of dry material. The ratio of cations to nitrogen (taken as nitrate ion) is also given in the table for each set of data, along with the approximate pH of plant juice.

The following points are clearly seen from the table:

(1) The total amount of nitrogen expressed in equivalents varied widely from 0·385 in the case of Timothy grass to 1·90 in the case of orchard grass, from 1·49 in the case of clover hay to 2·54 in cowpeas hay, and in differently manured wheat from 0·720 to 0·862. In spite of wide variation in the amount of nitrogen absorbed, there is a corresponding increase or decrease in the amounts of cations taken up by the plants of the same species and the ratio of cations to nitrogen all expressed in equivalents lies between 0·6 to 0·7 for a number of grasses, between 0·75 to 0·85 for legumes and for wheat between 0·36 to 0·39. Thus there is approximately a definite

TABLE II

Relationship between nitrogen (calculated as nitrate ion) and cations (Ca + Mg + K + Na) expressed in equivalents per 1,000 gm. of dry material

Plant	N	K	Mg	Na	Ca	Sum of cations	Cations /N	pH of plant juice	Source from where collected
					Grasses				
1 Rich pasture grass ..	·514	·172	·060	·010	·093	·335	·65	..	Jhonson (1900)
2 Young grass and aftermoth ..	·400	·113	·060	·022	·089	·285	·71	..	,,
3 Rye grass ..	·407	·151	·020	·023	·054	·248	·61	..	,,
4 Timothy grass ..	·386	·151	·035	·013	·061	·259	·67	..	,,
5 Blue grass ..	1·04	·331	·183	·056	·154	·724	·70	5·6*	Forbes (1913)
6 Timothy hay ..	·610	·145	·085	·138	·089	·457	·73	..	,,
7 Kentucky blue grass ..	1·520	·54	·15	..	·25	·94	·62	..	Archibald and Bennet (1935)
8 Orchard grass ..	1·90	·898	·242	..	·30	1·44	·76	..	,,
9 Red top ..	1·78	·670	·175	..	·31	1·155	·65	..	,,
10 Rhode grass ..	1·79	·57	·16	..	·38	1·11	·62	..	,,
11 Sheep fescue ..	1·49	·491	·125	..	·24	·856	·58	..	,,
12 Timothy ..	1·53	·624	·125	..	·25	·999	·66	..	,,
					Legumes				
1 Clover hay ..	1·49	·436	·225	·027	·571	1·259	·85	5·74*	Forbes (1913)
2 Alfalfa hay ..	1·65	·197	·308	·20	·523	1·218	·73	5·81*	,,
3 Soyabean hay ..	1·95	·406	·502	·057	·616	1·59	·80	5·8*	,,
4 Cowpeas hay ..	2·54	·206	·817	·281	·907	3·03	·86	..	,,
					Wheat				
1 Wheat (No manure 16 years av.)..	·862	·164	·063	..	·081	·308	·36	5·4†	Russel (1937)
2 Wheat manured 86 lb. per acre ..	·731	·176	·046	..	·064	·286	·39	,,	,,
3 Wheat ..	·720	·123	·071	·017	·062	·273	·38	..	Warington (1907)

* Data of Troug and Meacham (1919)

† Data of Atkins (1922)

and constant ratio between nitrogen and cations absorbed for each type of plant.

(2) Nitrogen taken as nitrate ion exceeds the sum of cations (Ca + Mg + K + Na), and

(3) Nearer the ratio of nitrogen to cations is to unity, greater is the pH value or less is the H-ion concentration of the plant sap. That is, in plants where the difference between nitrogen and cations absorbed is more, the H-ion concentration of the plant sap appears to be also more.

This supports the view held by Allison (1931) and Conrad (1934) that excess of nitrogen over the cations might have been absorbed as nitric acid and the presence of H ion is probably responsible for the excess of nitrogen over cations other than H.

The following evidence from Kappan (1918) further supports the statement made above. The roots of leguminous plants (with cations to nitrogen ratio 0·75 to 0·85) have a less acid sap than those of non-leguminous plants (whose cation nitrogen ratio is less than 0·75) in similar conditions.

It is to be however mentioned that the data on the pH of the plants as shown in Table II is meagre and has been collected from different sources. Although the differences in pH between each species is limited to a narrow range, undoubtedly in the words of Troug and Meacham (1919): "There is certain acidity which is most favourable for the life-process of that species".

Thus there is evidence to show that nitrification is important both from the points of view of solubilising the cations from the solid phase in the soil and also of their absorption by the plants.

The above view is justified from the following calculations made on field data. Studies in the changes of nitrate during growing season of wheat from a field experiment were conducted to compare the relative value of nitrogen in different organic manures and ammonium sulphate. Nitrate was found to be traces at flowering time and after harvest.

The following table gives the nitrate present at the time of sowing, the equivalent amount of cations accompanying the nitrate per acre and the total nitrogen recovered by the crop.

Nitrate and cation availability in soils

Treatment	NO_3N in mgm. per 1,000 gm. of soil	Nitrate N in lb. per acre	Cations accompanying nitrate lb. equivalents	Nitrogen recovered by crop in lb. equivalents		Approximate cations required by crop in lb. equivalents
				lb.	lb.	
1 No manure ..	11·4	22·8	1·62	31·9	2·3	0·87
2 Green manure sunhemp	15·3	30·6	2·20	32·1	2·3	0·87
3 Green manure cowpeas	16·2	32·4	2·32	32·3	2·2	0·84
4 Compost ..	12·0	24·0	1·72	35·2	2·5	0·95
5 F.Y.M. ..	10·0	20·0	1·42	35·4	2·5	0·95
6 Castor cake ..	12·7	25·4	1·82	31·7	2·2	0·84
7 Mustard cake ..	11·6	23·2	1·66	32·8	2·8	0·84
8 Ammonium sulphate ..	12·5	25·0	1·80	30·6	2·2	0·84

The amount of nitrate nitrogen present in the soil varied from 20 to 32·4 lb. per acre and the cations which accompany this nitrate expressed in lb. equivalents vary from 1·42 to 2·32. The cation requirements of the crop calculated from the nitrogen recoveries by mutiplying the nitrogen with the factor 0·39 (ref. Table II) are given in the last column. The cations required by the crop under different treatments varied from 0·84 to 0·95 lb

equivalents and as seen from the table can easily be met with by the cations that accompany the nitrates in the soil.

Discussion and Summary

The solvent which can possibly explain the increased availability of the mineral constituents to the plants, appears to be the nitric acid from the nitrification process that goes on in all the ordinary soils. The carbon dioxide evolved from the decomposition of the organic matter does not appear to have any serious effect on the soil minerals, although it is profusely liberated in soils. It may be that most of the carbon dioxide escapes as soon as it is produced and generally appears to be too weak to affect the highly buffered and stable system of soil.

Nitrate appears to have a definite role in the absorption of cations whatever may be the actual mechanism of absorption. The chemical composition of a number of plants indicates that there is a close relationship between nitrogen and cations absorbed (Ca + K + Mg + Na). The nitrogen absorbed is more than what can be accounted by the above cations and excess of nitrogen over cations appears to have entered along with H ion, in view of the fact the H-ion concentration of the plant sap is more in plants having wide difference of nitrogen and cations.

So far the availability of cations to the plants are concerned, field studies with wheat indicate that cations which accompany nitrate at the early growth period are sufficient to meet the requirements of wheat crop.

These experiments and calculations therefrom indicate that nitrification not only brings the cations into solution from the insoluble form but also acts as the dominant carrier of cations required by the plants and emphasises the need for taking into account the relationship between the nitrogen and the cations as shown above, in any mechanism for the absorption of the cations by the plant.

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STUDIES IN THE ADSORPTION OF BACTERIA

Part I. Adsorption of *B. subtilis* and *B. coli* by *Kaolin* and *Charcoal*

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Introduction

Adsorption is a familiar phenomenon which is associated with interfaces. It plays a very important part in determining the behaviour of colloidal systems and also living cells. It is thus of fundamental value in the study of Biochemistry as well as Bacteriology.

Several adsorption equations have been proposed by Gibbs, Freundlich, Langmuir, Brunauer, and others and an account of some of them has been given in an earlier paper. A new adsorption equation derived by Krishnamurti, *viz.*, $n = \frac{K}{1 + a \cdot e^{-\beta^c}}$ has been found to be applicable to the cases where the adsorption curves are S-shaped.

The adsorption of various substances—dyestuffs, acids and salts, bacteriophage, bactericides, by bacteria, has been investigated to some extent as it not only possesses theoretical importance but has much practical interest in Applied Bacteriology. Very little quantitative work appears to have been done on the adsorption of bacteria by various adsorbents.

The adsorption of bacteria by porous and powdery substances has been known for a long time. Observations on the adsorption of bacteria by various substances was reported by Kruger (1899) and by Eisenberg (1918). Some Russian workers (Chudiakow, Dianowa, Woroshilowa, Rubenschik and others) were specially interested in the application of the bacterial adsorption in soil investigations.

Rubentshick[1] and co-workers have examined the difference in the adsorption capacity of different kinds of mud. Gunnison and Marshal[2] have investigated the adsorption of *E. coli*, *C. welchii*, *L. acidophilus*, *Staphylococcus*, *B. subtilis* by kaolin, charcoal, Lloyd's reagent, $CaCO_3$, $BaSO_4$ and $Al(OH)_3$, following the usual observation that kaolin and charcoal are administered by mouth to persons suffering from intestinal bacterial disorders. They report that the concentration (Number per c.cm.) of organisms in suspension has no significant effect on adsorption.

If the adsorption of bacteria by well-known adsorbents—kaolin, charcoal, etc.—is of the usual type, it should follow one of the adsorption

isotherms. The object of this and the following papers in the series is to investigate quantitatively the adsorption of bacteria at the interfaces of various substances in order to examine the variation of the amount of adsorption with the concentration of the bacterial suspension, the specific nature of the absorbent and the bacteria, the area of the interface, temperature, addition of other solutions—colloidal or electrolytic, drugs, etc.

The results of the investigations on some aspects of this problem are reported in this paper.

Experimental

The bacteria employed in this investigation are *E. coli* and *B. subtilis*. They were cultured from specimens which were previously identified. Stock cultures were grown on sterilized Lemco-Agar slopes, and pure colonies were carefully scraped out from 2 or 3 days old slopes with a platinum loop and dispersed evenly in Normal saline solution so as to get a uniform count.

Equal volumes of bacterial suspension of increasing concentrations (increasing number of bacteria per c.cm.) were added to weighed amounts of purified and dried adsorbents, *viz.*, charcoal or kaolin, in thoroughly, cleaned test-tubes and shaken gently at intervals, in order to attain equilibrium and finally allowed to settle. When the adsorbent had completely settled down leaving a clear suspension on the top a measured quantity (·5 to 1·0 c.cm.) was taken out, diluted to 10 to 20 times its original volume and the number of bacteria present per one cubic millimetre was counted after introducing the suspension in the Spencer's improved American Hæmocytometer. The difference between this and the count from the original dispersion, kept in a test-tube under identical conditions but without the adsorbent, gives the number of adsorbed bacteria. A special research microscope was used for the investigation. In order to obtain consistent results, kaolin and charcoal were taken for investigation from the same stock samples, which were previously purified, dried and sieved.

Results and Discussion

Nature and mechanism of the adsorption of bacteria

The sizes of bacteria and of the kaolin and charcoal particles were examined with the help of the microscope using the object and the eyepiece micrometers.

The values are given below:

Bacteria (*B. subtilis*)	$1{\cdot}8\mu \times 0{\cdot}9\mu$
Charcoal particles	$1{\cdot}5\mu$ Diameter
Kaolin particles	$1{\cdot}1\mu$ Diameter

The sizes given above refer to the smallest of the charcoal and kaolin particles. In reality we found, after shaking with the bacterial suspension several larger aggregates of the charcoal and the kaolin particles containing varying numbers. It will be seen that we are dealing here with an interaction between bacteria and the particles of the adsorbent of near about the same size. In the studies of adsorption of molecules from the gaseous or liquid systems by powdery solid substances, we visualize a condensation of the molecules on the interface of the solid particles. In the present case which of the two phases constitutes the adsorbent or the adsorbate depends upon the size of its particles; for example, it has been found that when the bacteria were smaller than the aggregates of the charcoal particles the former arrange themselves as layers or clots round the charcoal or kaolin particles. We are then justified in regarding this as adsorption of bacteria on charcoal or kaolin. If on the contrary a bacterial suspension in pure water is mixed with ferric hydroxide sol, a mutual precipitation occurs, and an examination in the field of view of the ultra-microscope has revealed that ferric hydroxide particles are actually adsorbed on the surface of the bacteria. Thus the terms adsorbent and adsorbate are relative. The phenomenon here referred to corresponds to the mutual adsorption of colloidal particles.

The results of the adsorption and their interpretation

The results of the quantitative investigation of the adsorption of bacteria on charcoal and kaolin particles used as adsorbents at various concentrations of the bacterial suspensions commencing from a low value are given in Tables I, II, III, IV, and represented graphically in Fig. 1.

TABLE I

Adsorption of B. subtilis *on Animal Charcoal* (*see Fig.* 1, *Curve A*)

Volume of suspension added 5 c.c. Temperature 25° C.

Wt. of charcoal in gm.	No. of organisms per c.mm.		Total No. of organisms absorbed from the suspension	'a' Experimental	'a' Calculated from Krishnamurti's equation
	Before adsorption	After adsorption			
·0110	680×10^3	520×10^3	80×10^7	$7{\cdot}2 \times 10^{10}$	$7{\cdot}4 \times 10^{10}$
·0130	1360 × ,,	896 × ,,	232 × ,,	17·8 × ,,	23·0 × ,,
·0170	2040 × ,,	1000 × ,,	520 × ,,	30·5 × ,,	30·0 × ,,
·0180	2720 × ,,	1184 × ,,	768 × ,,	42·6 × ,,	40·5 × ,,
·0200	3400 × ,,	1420 × ,,	980 × ,,	49·0 × ,,	48·8 × ,,

TABLE II

Adsorption of B. coli *on Animal Charcoal* (*see Fig.* 1, *Curve B*)

Volume of suspension added 4 c.c. Temperature 30° C.

Wt. of charcoal in gm.	No. of organisms per c.mm.		Total No. of organisms adsorbed from the suspension	'a' Experimental	'a' Calculated from Krishnamurti's equation
	Before adsorption	After adsorption			
·0036	64 × 10^3	46 × 10^3	72·0 × 10^6	2·0 × 10^{10}	
·0036	192 × ,,	101·5 × ,,	362 × ,,	10·0 × ,,	16·2 × 10^{10}
·0034	320 × ,,	132 × ,,	752 × ,,	22·7 × ,,	20·6 × ,,
·0040	576 × ,,	244 × ,,	1328 × ,,	33·2 × ,,	32·8 × ,,
·0036	704 × ,,	328·7 × ,,	1501 × ,,	41·7 × ,,	40·0 × ,,
·0036	896 × ,,	488·5 × ,,	1630 × ,,	45·3 × ,,	46·0 × ,,
·0034	1120 × ,,	704 × ,,	1664 × ,,	48·9 × ,,	48·3 × ,,
·0040	1280 × ,,	790 × ,,	1960 × ,,	49·0 × ,,	48·6 × ,,

TABLE III

Adsorption of B. subtilis *on Kaolin* (*see Fig.* 1, *Curve C*)

Volume of suspension added 5 c.c. Temperature 25° C.

Wt. of charcoal in gm.	No. of organisms per c.mm.		Total No. of organisms adsorbed from the suspension	'a' Experimental	'a' calculated from Krishnamurti's equation
	Before adsorption	After adsorption			
·0170	560 × 10^3	512 × 10^3	24 × 10^7	1·4 × 10^{10}	1·5 × 10^{10}
·0110	1120 × ,,	996 × ,,	62 × ,,	5·6 × ,,	6·0 × ,,
·0120	1680 × ,,	1240 × ,,	220 × ,,	18·3 × ,,	18·3 × ,,
·0120	2240 × ,,	1320 × ,,	460 × ,,	38·0 × ,,	37·8 × ,,
·0130	3030 × ,,	1640 × ,,	690 × ,,	53·0 × ,,	52·9 × ,,

TABLE IV

Adsorption of B. subtilis *on Charcoal* (*see Fig.* 1, *Curve D*)

Volume of suspension added 4 c.c. Temperature 50° C.

Wt. of char coal in gm.	No. of organisms per c.mm.		Total No. of organisms adsorbed for the suspension	'a' experimental	'a' calculated from Krishnamurti's equation	'a' calculated from Freundlich isotherm	'a' calculated from Langmuir's isotherm
	Before adsorption	After adsorption					
·0042	$118{\cdot}2 \times 10^3$	81×10^3	150×10^6	$3{\cdot}5 \times 10^{10}$	$3{\cdot}6 \times 10^{10}$	$3{\cdot}5 \times 10^{10}$	$3{\cdot}5 \times 10^{10}$
·0050	237·0 × ,,	164 × ,,	293 × ,,	5·8 × ,,	5·3 × ,,	6·0 × ,,	6·6 × ,,
·0043	474·0 × ,,	363 × ,,	426 × ,,	9·9 × ,,	11·6 × ,,	14·8 × ,,	12·2 × ,,
·0048	711·0 × ,,	475 × ,,	944 × ,,	19·6 × ,,	16·2 × ,,	18·7 × ,,	14·7 × ,,
·0044	948·0 × ,,	680 × ,,	1070 × ,,	24·3 × ,,	23·5 × ,,	26·5 × ,,	17·7 × ,,
·0057	1185·0 × ,,	820 × ,,	1460 × ,,	25·5 × ,,	28·1 × ,,	32·1 × ,,	20·0 × ,,

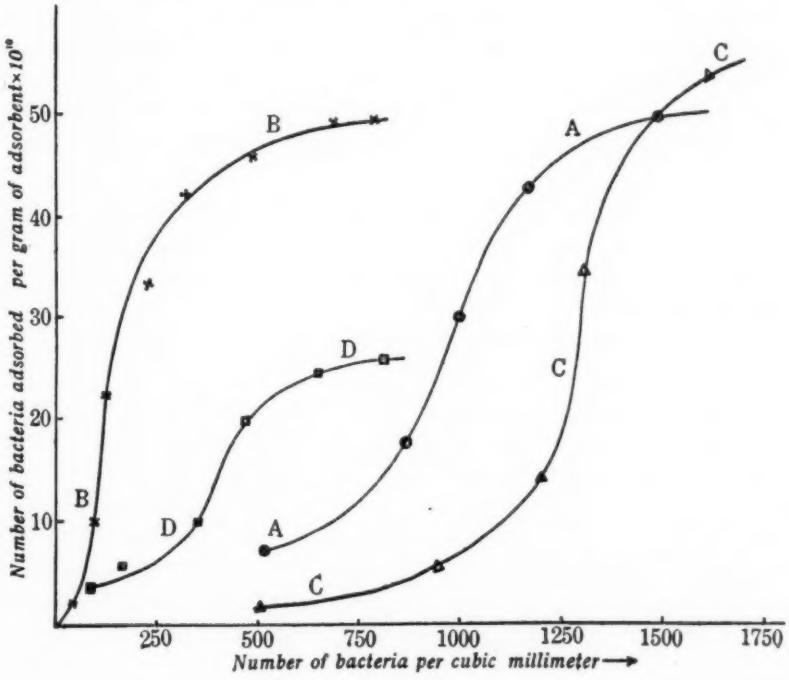

FIG. 1

It will be observed from the data given above that the adsorption of the bacteria by charcoal particles as indicated by 'a' (No. of bacteria adsorbed per unit mass) of the adsorbent is about the same order of magnitude varying between 2×10^{10} to 50×10^{10} and that the curves obtained by plotting the data are distinctly S-shaped. Further the nature and shape of the curves are reproducible showing that the S-shape is not an accidental occurrence but a characteristic feature of the phenomenon of adsorption encountered here. It will be also seen that the data do not fit in the well-known adsorption isotherms of Freundlich and Langmuir as is evident from a comparison of the observed data with the calculated values given in the last two columns of Table IV.

The view that the interaction between bacteria and the charcoal and kaolin particles is an adsorption process is supported by the following facts:—

(1) We have actually observed that when the bacteria in the course of their Brownian motion or motility approach the charcoal particles to within a distance of 1 μ they are drawn to the surface of these particles, indicating the existance of a surface attractive force. It is customary to associate surface phenomenon with adsorption, although owing to the comparatively larger size of the bacteria involved in this process and the infrequency of the close approach of the bacteria to within the range of attraction of the particles, the effect observed is not so pronounced as in the case of the molecules or ions.

(2) Another interesting point is that the nature of adsorption of bacteria round the charcoal particles and their aggregates as observed microscopically shows a marked similarity to the adsorption of colloidal particles on the surface of adsorbents.

(3) It will be seen from the discussion in a latter section of the paper that the retention of bacteria by charcoal or kaolin, both of which are very good adsorbents, is a very specific and a selective process reminding us of the adsorption of various enzymes, dyestuffs and other molecularly dispersed substances and indicating the chemical nature of the adsorption. Further, even between charcoal and kaolin the former has been found more efficient than the latter in agreement with the findings of previous workers in regard to the relative adsorptive capacity of charcoal.

(4) The S-shaped nature of the adsorption curves obtained by us show a striking similarity to the adsorption isotherm of water vapour on charcoal.[3] Such curves are quite familiar to workers in the field of adsorption. It is therefore natural for us to seek the interpretation of these

curves in terms of an adsorption process, rather than invoke the aid of an extraneous phenomenon.

(5) A striking observation made in the present investigation is that the amount of adsorption of bacteria increases with lowering of temperature —a fact clearly suggestive of an adsorption process.

We have observed that in some cases the bacteria formed a layer round the charcoal particles of the thickness of one cell, while in other cases—particularly at the stage of maximum adsorption—aggregates of cells were found lodged in between the adjoining charcoal particles. This was evidently due to the combined surface effects of the neighbouring charcoal particles causing the loss of mobility and the retention of the bacteria in between them. It is clear from this picture derived from the microscopic examination, that some of the bacteria initially adsorbed on the surface of the charcoal or kaolin particles, help in the further adherance of more bacteria,—an effect which is comparable to that observed in the case of the adsorption of substances like water vapour on charcoal. This may be regarded as a kind of " co-operative effect " where the presence of adsorbed molecules facilitate the adsorption of more molecules in neighbouring positions. Such a type of adsorption can rightly be represented by the equation proposed by Krishnamurti, *viz.*,

$$a = \frac{K}{1 + ae^{-\beta c}}.$$

Comparison of the observed values with those calculated with the help of this equation shows a close agreement which is satisfactory in view of the difficulties encountered in making accurate counts of bacteria. We are therefore evidently justified in regarding this expression as correctly reproducing the observed data, and therefore as one applicable to bacterial adsorption in general.

The study of the adsorption isotherms using charcoal as adsorbent at different temperatures reveal some interesting facts as may be seen from the data given in Table V and plotted graphically in Fig. 2.

The striking feature which one notices is that there is marked increase of maximum adsorption of bacteria with the lowering of temperature. Further a straight line is obtained by plotting the amount of adsorption of bacteria against 1/T, where T is the absolute temperature at which the experiment is carried out. From the slope of the curve one can draw some inference regarding the heat of adsorption.

TABLE V

Temperature of adsorption in degrees absolute	Maximum adsorption
298° K	50 × 10^{10}
302° K	49 × „
318° K	31·9 × „
323° K	25·5 × „

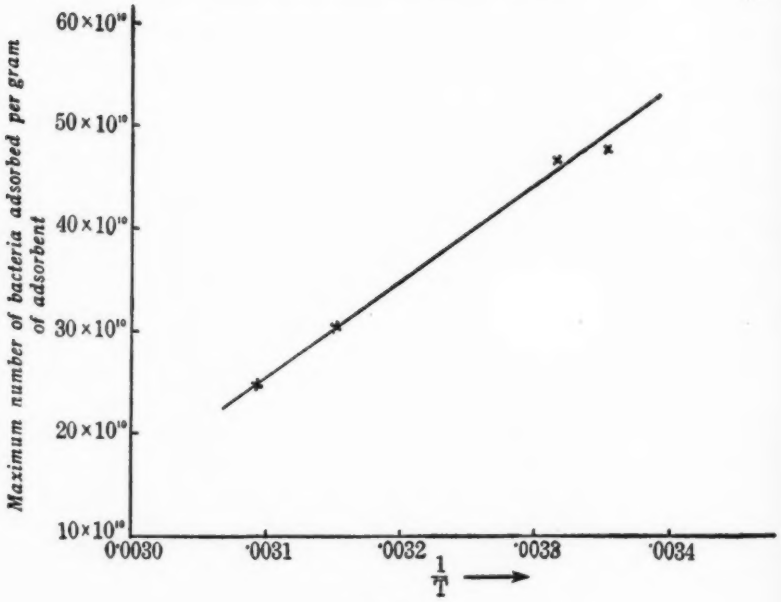

FIG. 2

It appears to us that the adsorption of bacteria is partly influenced by the adsorption of the enzymes and other proteins, which constitute an integral part of the surface of the bacterial cell, by the adsorbent.

Measurements of the electrophoretic velocities of the kaolin and bacterial suspensions have revealed that both the kaolin particles and the bacterial cells are negatively charged, although of the two the kaolin particles show higher migration velocities. What is surprising is that the mixtures of the same kaolin and the bacterial suspensions in different proportions indicate a decreased electrophoretic speed, which is less than that exhibited

by either of the pure suspensions. This can be seen from data in Table VI and seems to indicate that the electrical charge of the bacterial system is slightly diminished as a consequence of its adsorption by the kaolin particles thus facilitating the formation of aggregates of bacteria near the surface of the adsorbent.

TABLE VI

Suspension of	Velocity per minute per volt per cm.	Charge
Kaolin ..	0·275 mm.	Negative charge
B. Subtitis ..	0·0573 ,,	do
Mixture of kaolin and *B. Subtitis* ..	0·0343 ,,	do

The data bring out another interesting feature, *viz.*, that the addition of the bacterial suspension to that of kaolin causes a very marked decrease in the electrophoretic velocity of the kaolin particles and the migration velocity of the kaolin-bacteria complexes is reduced to about the same value as that of the bacteria. This observation clearly indicates that the surface of the kaolin particles is altered by the adsorption of bacteria converting it into one which closely resembles that of the bacteria.

The results further show that the adsorption of the bacteria by the kaolin particles is not a consequence of the electrical attraction of oppositely charged systems as that responsible for mutual coagulation of colloids. But it is probably due to the adsorption of the surface proteins and enzyme systems of the bacteria by the kaolin particles.

Separation of bacteria from mixtures by the adsorption technique

We have pointed out in the previous section of the paper that the adsorption of bacteria is a specific and selective process, being dependent upon both the nature of adsorbent and the bacteria used. A similar observation has also been made by some earlier workers.[1] But we have used this important feature of the adsorption process for evolving a new, simple, quick and delicate method for the separation of bacteria in a pure state from mixtures.

The method which has been developed by us is briefly as follows:—

A mixture of the suspension of two types of bacteria which could be easily distinguished from one another by their staining properties, was shaken periodically with a small amount of kaolin, which has been found

to possess a greater selective adsorptive capacity than charcoal until equilibrium was attained. Kaolin was allowed to settle and the supernatant liquid was gently taken out and relative count of the two bacterial species in it obtained by the usual microscopic method. In some favourable cases we have observed that starting with a mixture containing roughly equal numbers of the two types of bacteria the adsorbent removed one type completely, leaving the other type alone in the suspension above. But where this separation was not immediately achieved we had to resort to the method of " fractional adsorption ", by which we imply that the supernatant liquid obtained after the first treatment of the adsorbent was again treated in a separate test-tube with a fresh portion of the adsorbent and by repeating the process a number of times depending upon the nature of bacteria used only one bacterial species remained in the supernatant liquid in the final tube. This can be transferred to a suitable medium and cultured. This new method has been applied by us for the separation of:—

1. Staphylococci from *B. coli.*
2. *B. subtilis* from Staphylococci.

The method has to be standardised in order to make it useful in bacteriological technique.

Another interesting consequence of the selective nature of adsorption is in regard to the adsorption of bacterial enzymes. It is possible to so adjust the nature of the adsorbents as to suppress the activities of some of the enzymes in association with bacteria while the other enzymes will be allowed to display their usual activity. This subject is one of great importance and is being studied thoroughly.

Summary

1. The adsorption of *B. subtilis* and *B. coli* by two well-known adsorbents—kaolin and charcoal—has been quantitatively investigated at various concentrations of the bacterial suspensions. On plotting the data the total number of organisms adsorbed by unit mass of the adsorbent against the equilibrium concentration (number of organisms per c.mm. after adsorption)—typical S-shaped curves were obtained. This shape is a characteristic feature of the adsorption encountered here. It has been shown that the data obtained fits in the adsorption equation derived by Krishnamurti ($a = K/1 + ae^{-\beta c}$) far better than the adsorption isotherms of Freundlich and Langmuir.

2. The view that the interaction between bacteria and the charcoal and kaolin particles is an adsorption process is supported by several independent observations.

3. A marked increase of the maximum adsorption of bacteria has been observed with the lowering of temperature and a straight line is obtained by plotting the maximum amount of adsorption of bacteria against 1/T.

4. The specific and selective nature of the adsorption of bacteria by charcoal and kaolin has been successfully applied for evolving a new technique for the separation of bacteria from mixtures (*e.g.*, *B. subtilis* from Staphylococci). It is hoped that this method when standardized will be a useful addition to bacteriological technique.

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STUDIES ON FOLIAR SCLEREIDS IN DICOTYLEDONS

I. Structure and Ontogeny of Sclereids in the Leaf of *Diospyros discolor* Willd.

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Introduction

The importance of the study of foliar sclereids was recognised as far back as 1908 by Solereder, who pointed out their possible taxonomic value. Recent studies by Bailey and Nast (1944, 1945), Foster (1944, 1945 *a*), Subramanyam and Rao (1949) have confirmed their taxonomic significance in differentiating species within the genus. More interest, however, is being centred now on the ontogenetic development of sclereids, and important contributions by Foster (1945 *b*, 1947), Bloch (1946), Sterling (1947) and others have considerably advanced our knowledge about them. Recently the writer (1951) made an attempt to classify sclereids into different groups, based upon their origin from the epidermis, palisade or spongy cells.

Statements on the terminal, subterminal or diffuse types of distribution of sclereids are usually based on observations made on mature sclerieds in the leaf-tissue but it is only in a few cases that the early developmental stages have been studied. As pointed out by Foster (1946), the question whether the terminal sclereids originate from procambial cells in the developing veinlets or from adjacent cells of the ground meristem is of great interest. Recently, the finding of terminal as well as diffuse sclereids in the leaf of *Diospyros discolor* prompted me to make detailed observations on their origin and development.

Materials and Methods

The material for study was collected at the Government Botanic Gardens, Bangalore. *Diospyros discolor* is a medium-sized tree with large oblong coriaceous leaves. It is a native of the Philippine islands. Vegetative buds as well as small portions of young and old laminæ were fixed in formalin-acetic-alcohol. Microtome sections were stained by employing the tannic

acid-iron chloride-safranin method (Foster, 1934). Leaves were cleared and macerated as suggested by Foster (1946) and permanent mounts were prepared by the method outlined by Subramanyam and Rao (1949).

Distribution of Sclereids in the Mature Lamina

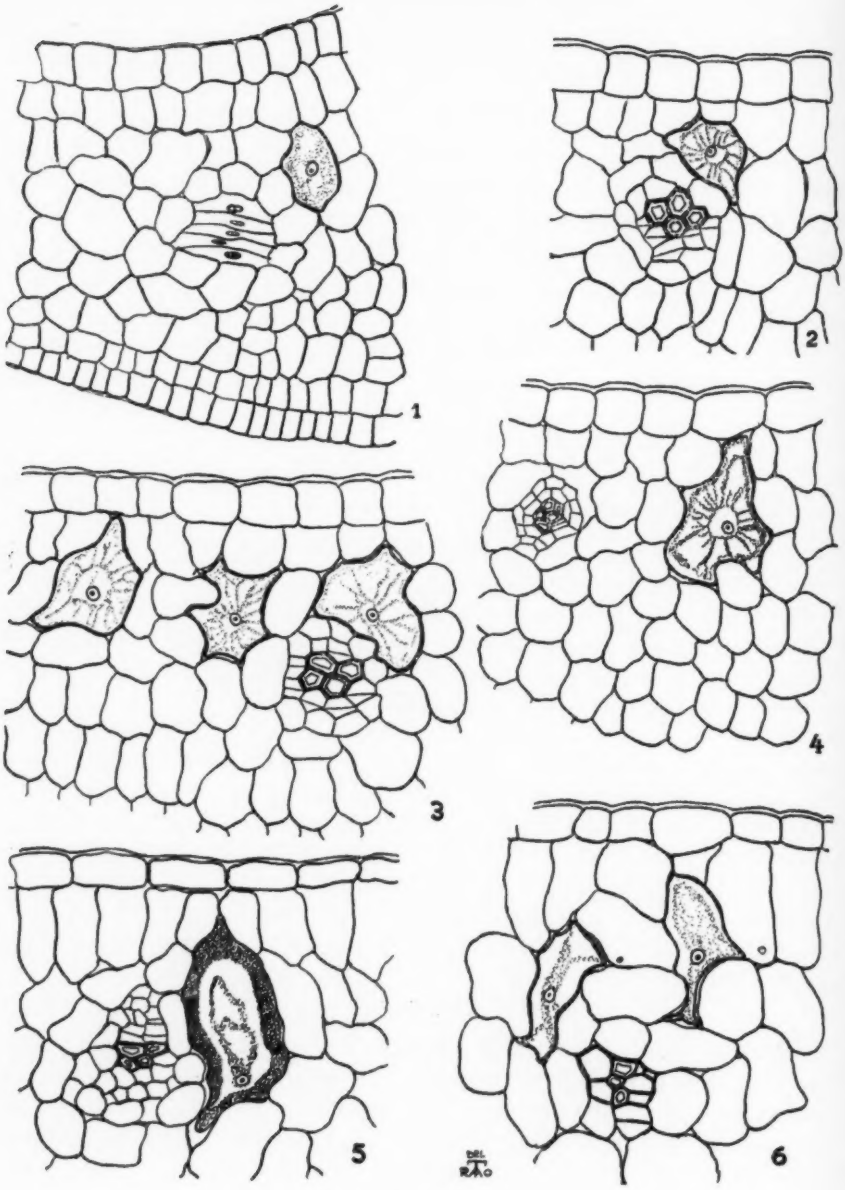
As described by Solereder (1908) the mesophyll of the mature lamina has a single layer of palisade cells on the adaxial side and a moderately lacunate spongy parenchyma. The smaller as well as larger veins are accompanied by well-developed sclerenchymatous cells.

One of the most salient features of the cleared leaf is the occurrence of sclereids both diffuse as well as in contact with the end of the veinlets (Plate IV, A–D). The sclereids are abundantly present near the midrib region. Diffuse sclereids are more numerous than the sclereids in contact with the tracheary elements of the veinlets. Exceptionally all the veinlets in a large vein-islet may be in close contact with the sclereids (Plate IV, A). Occasionally a portion of the tracheary vein or sclerenchyma or both may incurve towards the diffuse sclereid (Plate IV, A).

The study of macerated material and transverse sections of the mature lamina reveal that the sclereids are short prop-like ' cell forms ', distributed mostly in the palisade region and slightly protruding into the lacunar space (Figs. 12–13). The abaxial side of the sclereid, sometimes throws short branches extending into the lacunæ, whereas the adaxial side abuts close to the epidermal cells without undergoing any branching. On the whole, the sclereids do not show much variation in their shape, but differ slightly from each other in the possession of arrested plug-like branches at the abaxial tip. In no instance do the mature sclereids extend up and down so as to touch the two epidermal layers. Such form types are seen in the laminæ of *Linociera macrophylla* Wall, and *L. intermedia* Wight (Rao, 1950). In rare cases, on the adaxial side the mature sclereids slightly protrude between the radial epidermal walls and sometimes may even extend beyond them into the cuticle. Such a feature is reported in *Mouriria huberi* Cogn. (Foster, 1947).

Ontogeny

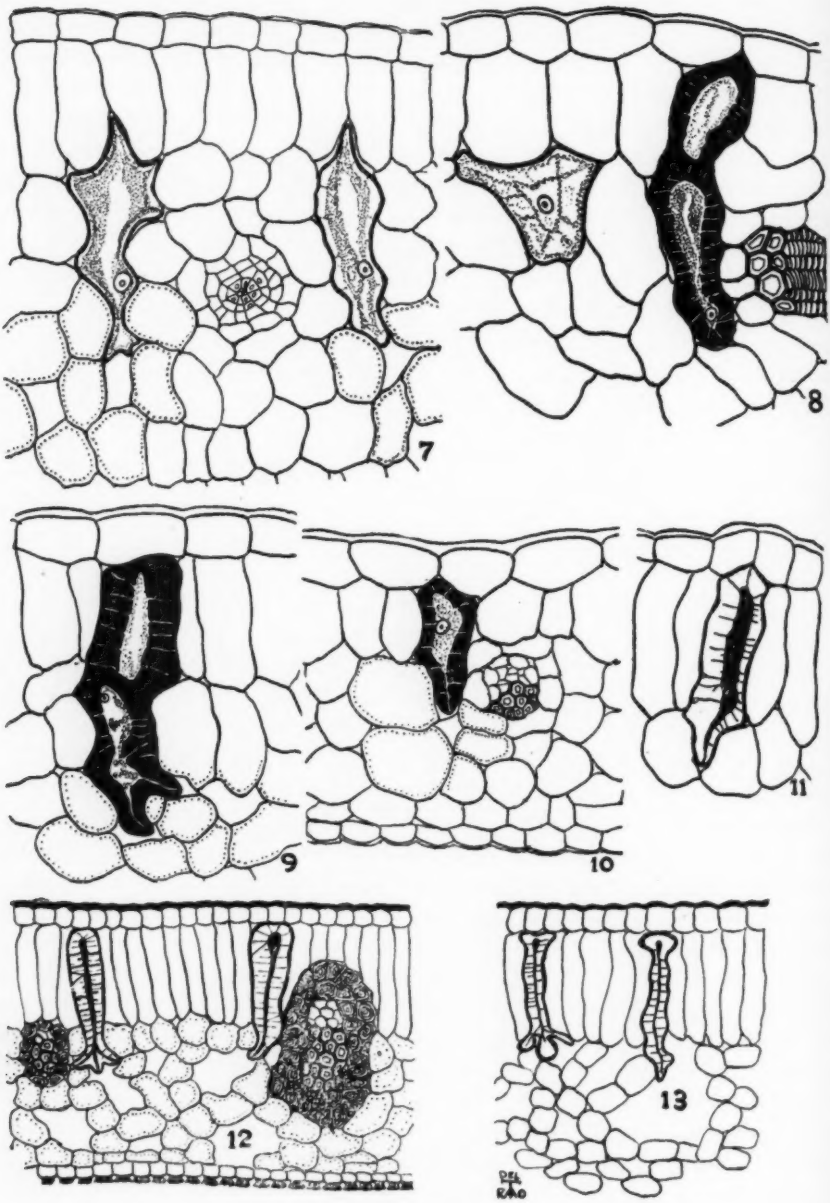
Developmental stages of the sclereids were traced in sections of young leaves taken from unfolding leaf-buds. In early stages the young lamina consists of 5 to 9 layers of closely arranged cells with small intercellular spaces. The vascular bundles are in an active state of differentiation, and a sheath of polyhedral cells is soon seen surrounding the xylem and phloem. The cutinised epidermis is clearly demarcated on the adaxial surface. At this stage the sclereid initials appear as large polyhedral cells, differ from



FIGS. 1–6

the adjacent cells by their slightly larger size, vacuolated cytoplasm and a prominent nucleus (Figs. 1–3). Such features have also been reported in *Mouriria huberi* (Foster, 1947). A careful examination of the lamina reveals that the sclereid initials originate from the cells of third layer situated beneath the upper epidermis. In the sub-marginal region they are in initmate contact with the differentiating vascular bundles. There was no evidence for a differentiation of the sclereid initials at the terminal portions of the procambial strand as reported in *Mouriria huberi* (Foster, 1947) and M*emecylon heyneanum* (Rao, in press). They are situated just above and outside the sheath of the vascular bundle. The enlargement of the sclereid initial is independent of the vascular differentiations in spite of its seemingly close contact with the vascular bundle in later stages. This reveals that the sclereid initial cell is a transformed spongy cell and not an integral part of the veinlet end.

In the course of development, the sclereid initial cells which are at first sub-spherical to polygonal in outline show a tendency to elongated in the vertical direction of the leaf. They may also throw small adaxial and abaxial processes (Figs. 5–6). These plug-like processes extend intercellularly between the walls of neighbouring cells. They make their way mostly in a vertical direction and very rarely in an oblique direction. The adaxial part of the growing sclereids enters the palisade region, which is well differentiated with a well-developed epidermal cells. Meanwhile the abaxial part grows downwards between the cells of the spongy parenchyma coming to a stop as it enters an air-lacuna (Fig. 7). The adaxial end of the sclereid does not show any branching. Only rarely small tube-like processes may extend between the walls of epidermal cells (Fig. 10). The abaxial end may, however, show slender dichotomous or trichotomous processes extending into the intercellular spaces (Figs. 8–9). In the final stage the abaxial processes do not fill up the intercellular space but they occupy a portion of the moderately big air lacunæ. Such a feature is reported in the petiolar sclereids of *Camellia* (Foster, 1944). As in *Camellia*, *Trochodendron*, *Mouriria huberi* (Foster, 1944, 1945 *b*, 1947), *Ternstroemia japonica* (Rao, in press) and *Olea* (Rao and Kulkarni, 1951) the sclereids of *Diospyros discolor* remain uninucleate although the nucleus may occupy different positions in the enlarging cell. In the final stage the sclereids develop a massive lignified wall leaving only a narrow lumen. The wall is homogeneous and possesses slender pit canals, which are often branched or forked as in *Camellia* (Fig. 11). The degenerating nucleus can be observed up to a late stage of lignification of the sclereid.



FIGS. 7–13

SUMMARY

The sclereids in the cleared mature leaf of *Diospyros discolor* show two types of distribution with reference to veinlets; either terminal or diffuse. Ontogenetic studies have, however, revealed that the initials of the seemingly terminal sclereids arise away from the procambial strands. They are in fact transformed cells of the spongy parenchyma and the terminal appearance is due to juxtaposed development. Sclereids should not be designated as " terminal " merely on the basis of their position in the mature leaf unless this is compared by an ontogenetic study.

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Explanation of Figures

Figs, 1–7. Transections through the submarginal portions of young laminæ showing early stages in sclereid ontogeny × 450 each.

Fig. 1. Very young sclereid initial cell in close contact with differentiating veinlet.

Fig. 2. Sclereid initial cell showing adaxial and abaxial processes.

Fig. 3. Idioblastic sclereid initial cell along with the sclereid initial cells in contact with the differentiating veinlet. Note the irregular shape and sharp processes of idioblastic sclereids.

Figs. 4–7. Sclereid initial cells of the expanded lamina of immature leaves, showing the prominent growth processes on the adaxial and abaxial sides.

Figs. 8–11. Transverse sections of nearly mature lamina showing advance stages in sclerid ontogeny × 450 each.

Fig. 8. Adult sclereid in close contact with the veinlets showing broad lumen and protoplast and nucleus.

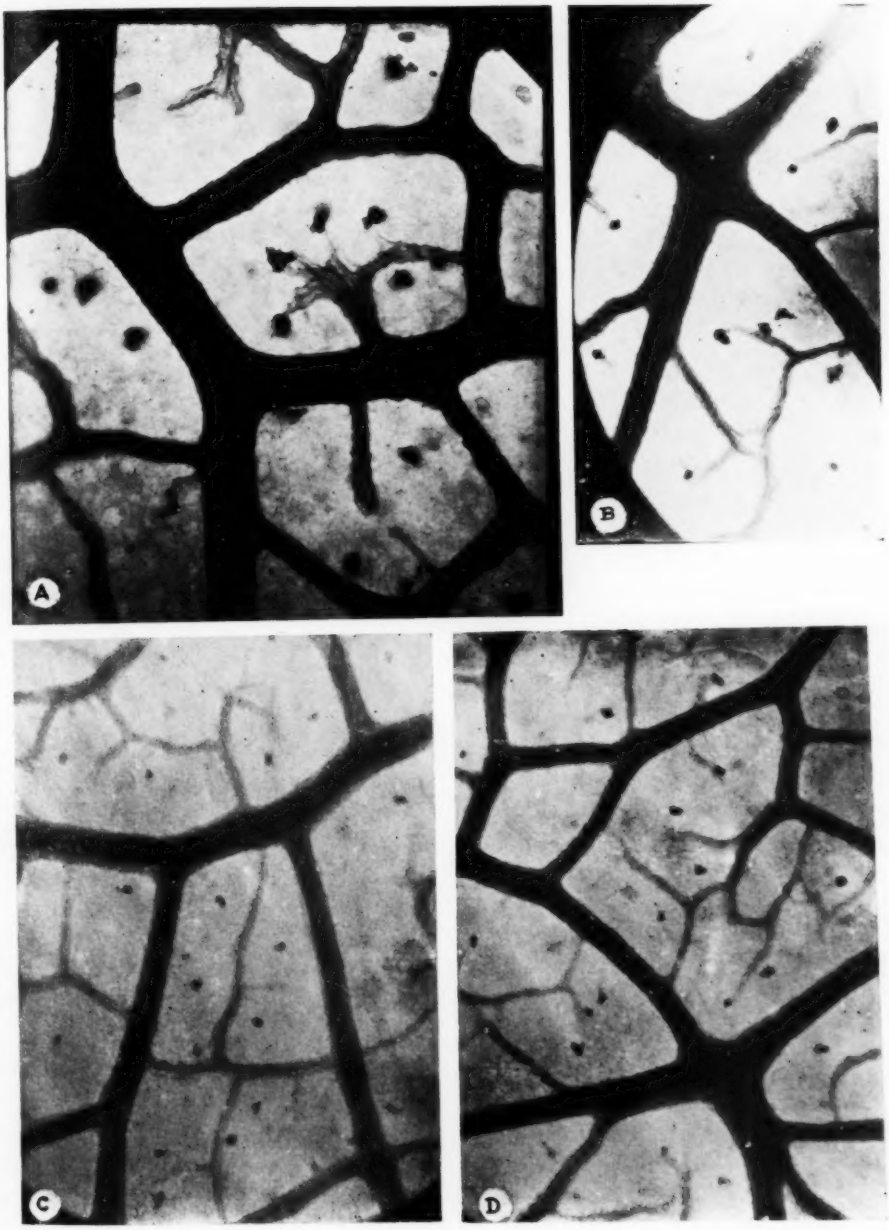
Fig. 9. Adult diffuse sclereid with forked abaxial process lying in a small air space.

Fig. 10. Near the margin — Adult pseudo-terminal sclereid.

Fig. 11. Adult idioblastic sclereid with channel-like lumen and forked pit canals.

Figs. 12–13. Transections of mature lamina showing pseudo-terminal & idioblastic sclereids. Note the branched or unbranched abaxial processes occupying a small portion of the air-lacuna.

A to D — Cleared lamina of *Diospyros discolor* showing the occurrence of diffuse sclereids as well as sclereids in contact with the end of veinlets, × 100 each.


A
B
C
D

IMPORTANCE OF MANURING IN POTATO CROP RAISED FROM CUT PIECES

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Introduction

The use of small seed pieces and even single eyes in the propagation of potato is known from a long time but their practical application in commercial farming has evoked interest only recently. The problem has gained particular interest during the war when food production and economy was as important as the actual war.

In U.S.S.R. the Government ordered a big increase in potato crop at the beginning of war but the seed supply proved to be the limiting factor. Lysenko and his co-workers (Anon, 1942) met this emergency by utilizing as seed a small piece from the 'rose' end of the tuber and using the rest of the potato for food. It is said that the top yielded as well as the whole tuber. Similar emergency methods have been discussed by Copisarow (1943) as a means of war economy. Pal and Deshmukh (1944) have also examined the possibility of using chips in the cultivation of potato. The 'eyes' and 'tops' have given lower yields than the whole tuber. The yield from 'tops' is, however, not very unsatisfactory. Sen and Chakravarti (1945) have reported similar results. Apart from economy of food, Evans (1943) studied the problem for saving shipping weight and to transport the 'seed' chips by air to some of the overseas British dependencies which could not receive timely supplies due to war-time vehicular difficulties and the seed that reached was spoilt and too late for the planting season. 'Chips' are reported to have given satisfactory yields.

In other countries, work on the use of 'chips' in the cultivation of potato was undertaken as a war-time emergency measure but in India, the problem of seed economy is of a permanent importance. Seed supply has been a limiting factor in the cultivation and extension of potato even in normal times and ever since the break of war it has become much more acute. Foreign imports of seed are completely closed and the country is thrown entirely upon its own resources which fall short of the total demand. But

more important than shortage is the need of reducing seed costs per acre. The prices are soaring high and the Indian ryot is too poor to afford such heavy initial investments.

How to economise the use of seed without sacrificing the yield? This and allied problems are under investigation at this Research Station since 1944 and important results have been obtained (Kapoor, 1949, 1950). Size of seed is an important factor in determining yield and workers attribute the decreased yield from a small seed piece to the relatively small amount of stored food in the mother tissue. Nutrition being an important conditioning factor of plant growth, advantage is taken in the present investigation to determine if heavy manuring could make up the depreciating effect of low food reserve in the small seed piece.

Methods

Experiment was conducted in the years 1945–46 and 1947–48. Sowing was done in cement concrete pots of 12 × 15″ size containing soil and soil-manure mixtures. Since field experiments are subject to multiplicity of factors, the present work was preferably planned as pot culture where variations due to soil, irrigation and interculture could be controlled more effectively. At the same time it appeared essential to confirm the results of pot culture under field conditions also. Another experiment (Kapoor, 1950) on similar lines and relating to the influence of seed piece, spacing and manuring was, therefore, laid out in a field of size 234 × 189′. It was a replicated randomised blocks design.

Seed Material

Since certified potato seed is not available in India (Ramanujam, 1947), great care had to be exercised in the selection of initial seed material. Variety *Darjeeling Red Round* which is popularly grown in this locality and is sown as cut pieces, was used for the inquiry. Seed was obtained through the Directorate of Agriculture, West Bengal. A rigorous selection was made to obtain potatoes of similar shape and other tuber characters. All diseased and damaged tubers were discarded.

Treatments

The experiment included 3 variables with 3 levels under each. These were:

1. Levels of manuring 3, *viz.*, M_1—field soil only; M_2—field soil + $\frac{3}{4}$ lb. of cowdung manure and 1·0 gm. of amm. sulph. per pot; M_3—field soil + $2\frac{1}{2}$ lb. of cowdung manure and 2·0 gm. of amm. sulph. per pot.

Cowdung manure in weighed quantities was mixed 20 days before sowing in the top 4–5 inches soil of each pot.

For this experiment, cowdung manure was selected in preference to artificials since it is a complete manure and besides humus and minor elements, it supplies all the essential elements, *viz.*, N, P and K for the proper growth of potato plant. This was supplemented by an application of ammonium sulphate in order to increase the quantity of N which is in deficiency in the soil. It may be stated here that little systematic work on the manuring of potato exists in India at present. Experiments on the manurial requirement and on the balanced nutrition of potato by application of N, P and K at various levels, with and without farm yard manure are in progress at this Research Station.

2. Sizes of seed piece, 3, *viz.*, P_1—quarter tuber; P_2-half tuber; P_3—whole tuber.

3. Sizes of seed tuber 3, *viz.*, T_1—20 gm. weight; T_2—40 gm. weight; T_3—80 gm. weight.

The total number of treatment combinations amounted to 27 and were obtained by all combinations of the above 3 factors.

The number of replications, pots per treatment combination and other relevant details are given below:—

	1945–46	1947–48
Number of replications	3	5
No. of pots per treatment combination per replication	10	5
Total No. of pots for the experiment ..	810	675
Date of sowing	23–11–45	10–12–47
Date of harvesting	23–3–46	31–3–48

As stated earlier, the main results of the experiment were confirmed by laying out a similar trial on a larger-scale in the field as well.

Experimental Findings

Observations have been made on germination, growth, yield and grade of the produce. The present paper, however, is restricted to the effects on more important characters of yield and grade only.

Yield of Tubers per Hill

Yield of tubers is presented in Table I. A good range of variation is noticed in the different treatment combinations. The lowest yield is given

TABLE I

Yield of tubers per hill in gm. under the 27 treatment combinations of manuring (M), seed piece (P) and seed tuber (T), 1945–46

	T_1			T_2			T_3		
	P_1	P_2	P_3	P_1	P_2	P_3	P_1	P_2	P_3
M_1	58·0	76·8	85·2	73·0	81·4	93·6	78·2	93·4	124·6
M_2	142·8	172·5	192·7	167·6	185·1	211·6	167·4	207·8	236·8
M_3	287·8	298·7	301·8	293·2	301·6	300·8	301·8	302·6	318·8

Critical difference at 5% = 18·80

by the quarter piece prepared from small tuber and grown in M_1 level of manuring ($M_1P_1T_1$) and the highest is recorded from the whole tuber of 80 gm. weight grown in M_3 fertility level ($M_3P_3T_3$).

Main Effects.—Maximum response is made to the rate of manuring (Table II). M_1 produces a low yield of 84·97 gm.; the yield increases by 102·19 gm. when manuring level is raised to M_2 and a further increase of 113·65 gm. is noticed when rate of manuring is increased to M_3. The increases are highly significant. Yield is also affected by the size of seed piece. Whole tuber yields 207·36, half piece 191·13 and quarter piece 174·45 gm. per hill. The increase in yield of P_3 over P_2 and of the latter over P_1 is 16·23 and 16·68 gm. respectively. Effect of size of seed tuber is quite similar to that of seed piece, *viz.*, large tuber is associated with high yield and small seed tuber with low yield. Large seed tuber yields 203·51, medium 189·80 and small 179·63 gm. of tubers per hill.

Interactions.—Data pertaining to first order interactions are recorded in Table II. Size of seed piece affects the yield of tubers but the degree of effectiveness varies under the different levels of manuring. Increase in yield of P_3 over P_1 is 31·37 under M_1, 54·47 under M_2 and 12·86 gm. under M_3 level. Increases under the first two levels are highly significant while it is barely so at M_3 level. The size of set, therefore, which is important in determining the yield of potato under low and medium manuring remains hardly so when the fertility level is kept high.

Interaction of size of seed tuber with levels of manuring shows a trend similar to that of size of seed piece, *i.e.*, the seed tuber size which induces great differences in yield under M_1 and M_2 levels does not do so under the third level of manuring. Increase of large over small tuber under the three

TABLE II

Yield of tubers per hill in gm. under the main effects and first order interactions of manuring (M), seed piece (P) and seed tuber (T), 1945–46

	P_1	P_2	P_3	Mean	Increase
M_1 ..	69·80	83·93	101·17	84·97	
M_2 ..	159·26	188·48	213·73	187·16	+102·19
M_3 ..	294·31	300·97	307·17	300·81	+113·65
Mean ..	174·45	191·13	207·36		
Increase ..		+16·68	+16·23		

	T_1	T_2	T_3	Mean	Increase
M_1 ..	73·40	82·71	98·80	84·97	
M_2 ..	169·35	188·13	204·00	187·16	+102·19
M_3 ..	296·15	298·57	307·73	300·81	+113·65
Mean ..	179·63	189·80	203·51		
Increase ..		+10·17	+13·71		

	P_1	P_2	P_3	Mean	Increase
T_1 ..	162·91	182·71	193·28	179·63	
T_2 ..	177·97	189·40	202·04	189·80	+ 10·17
T_3 ..	182·48	201·28	226·75	203·51	+ 13·71
Mean ..	174·45	191·13	207·36		
Increase ..		+16·68	+16·23		

S.E. of increases	– Main effects ..	= 3·11
S.E. of increases	– 1st order interactions ..	= 5·38
C.D. at 5%	– Main effects ..	= 6·22
C.D. at 5%	– 1st order interactions ..	= 10·76

levels is 25·40, 34·65 and 11·58 gm. respectively; the increase of 11·58 is insignificant at 1% level. Thus the seed tuber also, similar to size of seed piece, loses its effectiveness at high level of fertility.

Interaction between size of seed piece and seed tuber indicates that the size of seed piece remains effective irrespective whether a small, a medium or a large seed tuber is used and likewise the size of seed tuber induces substantial differences under all the sizes of seed piece. Thus the increase in yield of P_3 over P_1 is 30·37, 24·07 and 44·27 gm. under small, medium and large seed tuber and the increase of T_3 over T_1 is 19·57, 18·57 and 33·47 gm. under quarter, half and whole piece respectively. The increases are all significant.

Number of Tubers per Hill

Tuber count per hill has shown great variations ranging from 5·00 to 16·33 in the different treatments. Data are presented in Table III.

TABLE III

Number of tubers per hill under the 27 treatment combinations of manuring (M), seed piece (P) and seed tuber (T), 1945–46

	T_1			T_2			T_3		
	P_1	P_2	P_3	P_1	P_2	P_3	P_1	P_2	P_3
M_1 ..	5·00	7·00	8·00	6·00	7·46	9·40	6·46	8·33	11·60
M_2 ..	7·93	10·00	12·00	9·46	10·93	13·20	9·86	13·13	16·13
M_3 ..	13·20	13·53	14·00	13·46	14·00	14·53	14·20	14·53	16·33

Critical difference at 5% = 0·96

Main Effects.—The number of tubers changes when a change is made in the level of manuring, size of seed piece or size of seed tuber (Table IV). The greatest effect is noticed under the manuring factor and number increases with increase in the rate of manuring, being 7·69, 11·40 and 14·19 per hill under the three levels respectively. The increases are all highly significant. Effect of size of seed piece is not as great as that of manuring. The increases, however, are quite significant. When a quarter piece is sown the number of tubers is 9·50 but the number rises to 12·79 per hill when a whole tuber is planted. Differences recorded under variations of size of seed tuber though significant are relatively small. Increase of large over medium and of the latter over small seed tuber is 1·34 and 0·87 respectively.

Interactions.—First order interactions between levels of manuring, size of seed piece and size of seed tuber are presented in Table IV. Effect of size of seed piece is more pronounced when manuring level is low than when it is high. The whole tuber shows an increase of 1·33 over the quarter piece under M_3 compared to 3·84 under M_1 level. The increases however are all significant.

Interaction of manuring with size of seed tuber is practically of the same nature as with the size of seed piece. Accordingly, the increase in tuber number caused by using a larger seed tuber is less under M_3 compared to M_1 level.

TABLE IV

Number of tubers per hill under the main effects and first order interactions of manuring (M), seed piece (P) and seed tuber (T), 1945–46

	P_1	P_2	P_3	Mean	Increase
M_1 ..	5·82	7·60	9·66	7·69	
M_2 ..	9·08	11·35	13·77	11·40	+3·71
M_3 ..	13·62	14·02	14·95	14·19	+2·79
Mean ..	9·50	10·99	12·79		
Increase ..		+ 1·49	+ 1·80		
	T_1	T_2	T_3	Mean	Increase
M_1 ..	6·66	7·62	8·80	7·69	
M_2 ..	9·97	11·20	13·04	11·40	+3·71
M_3 ..	13·57	14·00	15·02	14·19	+2·79
Mean ..	10·07	10·94	12·28		
Increase ..		+ 0·87	+ 1·34		
	P_1	P_2	P_3	Mean	Increase
T_1 ..	8·71	10·17	11·33	10·07	
T_2 ..	9·64	10·80	12·37	10·94	+0·87
T_3 ..	10·17	12·00	14·68	12·28	+1·34
Mean ..	9·50	10·99	12·79		
Increase ..		+ 1·49	+ 1·80		

S.E. of increases	– Main effects	..	= 0·15
S.E. of increases	– Ist order interactions	..	= 0·26
C.D. at 5%	– Main effects	..	= 0·30
C.D. at 5%	– Ist order interactions	..	= 0·52

Interaction between size of seed tuber and seed piece indicates that both the factors are important in inducing differences in the tuber number. Increase of size of set under all the sizes of seed tuber produces an increase in the number of tubers per hill. A similar rise is also noticed under all sizes of seed piece when the seed tuber is changed from small to large.

Average Size of Tuber

Besides affecting the various growth and yield attributes, the factors have also induced important variations in the grade of produce. Table V presents the relative effects on the size of potato tuber.

Main effects.—Maximum effect is shown by the manuring factor (Table VI). Tuber size is small under M_1 and it increases with increase in the level of manuring, becoming almost double in size at M_3 level. The

TABLE V

Average size of tuber in gm. under the 27 treatment combinations of Manuring (M), seed piece (P) and seed tuber (T), 1945–46

	T_1			T_2			T_3		
	P_1	P_2	P_3	P_1	P_2	P_3	P_1	P_2	P_3
M_1 ..	11·61	10·97	10·65	12·17	10·90	9·95	12·10	11·21	10·76
M_2 ..	17·90	17·26	16·07	17·71	16·92	16·01	16·94	15·82	14·67
M_3 ..	21·80	22·07	21·50	21·77	21·53	20·70	21·24	20·81	19·50

Critical difference at 5% = 0·64

TABLE VI

Average size of tuber in gm. under the main effects and first order interactions of manuring (M), seed piece (P) and seed tuber (T), 1945–46

	P_1	P_2	P_3	Mean	Increase
M_1 ..	11·96	11·03	10·45	11·15	
M_2 ..	17·52	16·67	15·58	16·59	+5·44
M_3 ..	21·60	21·47	20·57	21·21	+4·62
Mean ..	17·02	16·39	15·53		
Increase ..		−0·63	−0·86		
	T_1	T_2	T_3	Mean	Increase
M_1 ..	11·08	11·01	11·36	11·15	
M_2 ..	17·08	16·88	15·81	16·59	+5·44
M_3 ..	21·79	21·33	20·52	21·21	+4·62
Mean ..	16·65	16·40	15·89		
Increase ..		−0·25	−0·51		
	P_1	P_2	P_3	Mean	Increase
T_1 ..	17·10	16·77	16·07	16·65	
T_2 ..	17·22	16·45	15·55	16·40	−0·25
T_3 ..	16·76	15·95	14·98	15·89	−0·51
Mean ..	17·02	16·39	15·53		
Increase ..		−0·63	−0·86		

S.E. of increases – Main effects .. = 0·09
S.E. of increases – 1st order interactions .. = 0·18
C.D. at 5% – Main effects .. = 0·18
C.D. at 5% – 1st order interactions .. = 0·36

size is 11·15, 16·59 and 21·21 gm. under the three levels respectively. Differences are highly significant. The nature of effect of size of set is different than that of manuring. Increase in size of set lowers the size of potato tuber while increase in manuring causes a considerable improvement. The average weight of tubers under the quarter, half and full seed tuber is 17·02, 16·39 and 15·53 gm. respectively. Critical difference is 0·18. Response to size of seed tuber is similar to that of seed piece except that the differences are relatively small. The use of small seed tuber yields a better grade of potatoes, a fall of 0·25 gm. is noticed under the medium size seed tuber and a further fall of 0·51 gm. is recorded when the large seed tuber is planted. Decreases are significant.

Interactions.—First order interactions are presented in Table VI. Effect of size of seed piece remains unaltered whether the manuring level is low or high. Large seed piece compared to the small one continues to yield poorer grade of potatoes under all the three levels of manuring.

Response to size of seed tuber is also similar. Planting of large seed tuber is associated with the production of small-sized potatoes and it remains so irrespective of the rate of manuring.

The study of interactions between size of seed piece and seed tuber indicates that both maintain their individual effect under all the levels of the other factor. Large seed piece compared to the small one yields relatively small-sized potatoes whether the seed tuber used for making the sets is small, medium or large. Likewise, the large seed tuber compared to the small one yields potatoes of comparatively small size whether it is sown as quarter, half or whole tuber.

Results obtained in the year 1947–48 are in general agreement with those detailed above.

Summary and Conclusions

The present paper deals with the results of an experiment on the influence of manuring, size of seed piece and size of seed tuber on the yield and grade of potato. The total number of treatment was 27 and was obtained by all combinations of the following factors.

1. Levels of manuring, 3 *viz.*, M_1—field soil only; M_2—field soil + ¾ lb. of cowdung manure and 1·0 gm. of amm. sulph. per pot; M_3—field soil + 2½ lb. of cowdung manure and 2·0 gm. of amm. sulph. per pot.

2. Sizes of seed piece 3 *viz.*, P_1—quarter tuber; P_2—half tuber; P_3—whole tuber.

3. Sizes of seed tuber 3, *viz.*, T_1—20 gm. weight; T_2—40 gm. weight; T_3—80 gm. weight.

Effects on yield and number of tubers per hill and average size of tuber are recorded.

2. Manuring has produced a marked effect on all the three attributes. Low manuring gives poor yield, less number and small size of potatoes as against high yield, large number and bigger size under heavy manuring. Increase in size of seed piece improves the yield and number of tubers per hill but reduces the average size of tuber. As regards size of seed tuber its effect is precisely the same as that of the size of seed piece.

3. Influence of size of seed piece differs with the level of manuring in the soil. Under M_1 level the quarter seed piece gives a significantly lower yield than the whole set but the difference in yield under M_3 is very small. A similar effect is noticed on the number of tubers per hill. As regards its influence on the size of potatoes, it remains unaltered whether the manuring level is low or high; large seed piece continues to yield poor grade of produce.

4. Interaction between size of seed tuber and manuring shows a trend similar to that of size of seed piece.

5. Interaction between size of seed piece and seed tuber indicates that the size of seed piece remains effective irrespective whether a small, medium or a large seed tuber is used and likewise the size of seed tuber induces marked differences under all the sizes of seed piece.

6. The possibility of using small sets, whole or cut, under heavy manuring is brought out. Seed can be economised without materially affecting the yield of potato crop.

Acknowledgements

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FERTILIZATION VALUE OF TANK SILTS

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The ameliorative influences of silts as fertilizers in husbanding impoverished or effete soils has been known for decades; in certain parts of the world, silts carried by rivers are the only means of fertilization to the soil. The practice of incorporating silt into nursery bed soils for raising rice seedlings is followed in China, Japan and elsewhere. It is well known that silts contain valuable organic matter and provide the basic nutritional factors for augmenting plant growth, similarly, the value of sewage sludge as organic manure is a recognized fact. It must be mentioned, however, that silts occurring in different environs are bound to vary in their chemical composition and fertilizing value (Mukerji, 1923; Waksman, 1938; Hoon and Dhawan, 1944). According to Hoon and Dhawan (1944) silts carried by the rivers of the Punjab contained very small quantities of nitrogen, phosphates and potash and thus could not be regarded as direct fertilizers. Much work has been done in evaluating the fertilizing value of sewage sludge (Brouwer, 1941; Joshi, 1945; Stephenson and Bollen, 1946); according to Stephenson and Bollen (1946) the chemical composition of the sludge investigated by them was equal to that of farm-yard manure, although Hall (1929) states that the action of sludge as manure is very small. The overall beneficial effects of silts and sewage sludge on plant growth is a recognized fact but very little is known as to how exactly the constituents of these organic manures influence plant growth.

The experiments reported here and carried out in pot culture studies under glasshouse conditions were planned essentially for providing preliminary gleanings in understanding the fertilizing value of tank silts. The problem was very kindly suggested by Prof. T. S. Sadasivan, M.Sc., Ph.D. (London), Director, University Botany Laboratory, Madras, and was taken up at the instance of the Collector of Madras.

Materials and Methods

The silt samples used in these investigations were collected from Chitrakulam (sample A) and Kapaleswar (sample B) tanks, Madras City, and were supplied by the Collector of Madras. Rice was selected as the indicator plant in evaluating the status of these silts as fertilizers mainly because the index of growth response of rice to manures and fertilizers is easily amenable to logical interpretation. The percentage of moisture in both the silt samples was determined at the time of experimentation by standard methods (A.O.A.C., 1945). Both silt samples were incorporated into sieved garden soil (sandy loam) to give concentrations of 5, 10, 15, 20, 25, 50, 75 and 100 per cent. (pure silt) on dry weight basis. The silt soil mixtures were put into earthenware pots of $6 \times 5 \times 3$ size at the rate of 500 g. per pot. In all 5 pots were maintained at every concentration for each of the silt samples; similarly, 5 pots containing garden soil alone served as control. Rice seeds at the rate of 25 seeds per pot were sown by first removing 100 g. of the upper soil layer, spreading the seeds equidistant from one another and finally covering up with the removed soil. The pots were watered daily and the entire experiment incubated under glass-house conditions for 3 weeks; after this period the seedlings in the pots were removed carefully and measurements of shoot length and dry weight determinations were conducted, taking into account 15 seedlings in all, 3 seedlings being taken from each replicate.

Table I

Showing Influence of Various Concentrations of Silt on Shoot Length and Dry Weight of Rice Seedlings

(taken three weeks after sowing)

Percentage concentration of silt in garden soil	Shoot length in inches (mean of 15 readings)		Dry weight of shoots of 15 seedlings (in mg.)	
	Sample A	Sample B	Sample A	Sample B
5	10·5	8·0	256	187
10	10·6	10·7	264	236
15	11·7	11·8	284	324
20	11·5	11·7	256	270
25	11·5	11·6	255	265
50	11·5	11·6	242	262
75	11·6	11·6	245	255
100	11·5	11·7	240	260
Control ..	7·6	7·6	184	184

TABLE II

Showing Percentage Content of Nitrogen and Potassium in the Silt Samples and in Garden Soil

(calculated on dry weight basis)

Sample	Percentage moisture	Percentage nitrogen	Percentage potassium
Sample A ..	80	356 mg.	426 mg.
Sample B ..	46	230 mg.	316 mg.
Garden soil ..		116 mg.	245 mg.

Chemical assays of the silt samples were carried out as follows: Nitrogen was estimated employing a modification of the method of Narayanayya and Subramanian (1935), the modification being essentially in the direction of absorbing the evolved NH_3 in 2 per cent. boric acid and back titration of NH_3 with standard HCl using Ma Zuzaga reagent. To estimate potassium the silt was first ignited and extracted as described by Rajagopalan (1939). Iron and other bases were then precipitated as recommended by Bouser (Narayanayya and Subramanian, 1935), and filtered off; potassium was then estimated in the filtrate by the method of Wilcox (1937). Results are incorporated in Tables I and II.

DISCUSSION OF RESULTS

The manifestation of fertilizing value of the silt samples in catalyzing seedling growth in rice was very striking as shown by Plate V and Tables I and II. Addition of silt even at such low concentrations as 5 and 10 per cent. contributed to considerable increase in shoot length and dry weight of seedlings, this being more marked in the case of sample A wherein at 5 per cent. level the shoot length increased by about 3 inches over that of the control; the corresponding figures of dry weights were 256 mg. and 184 mg. respectively (Table I). However, in the case of sample B at 5 per cent. concentration no appreciable increase in dry weight or shoot length was observed (Table I). This may be explained by the fact that sample A contained 126 mg. per cent. of nitrogen in excess of that contained in sample B (Table II), whereas in the former the percentage nitrogen present was more than three times that present in ordinary garden soil, in the latter it was only about twice as much. The corresponding values of potassium showed that sample A contained considerably more of potassium than sample B, although both the samples were very rich in this element as compared to garden soil (Table II).

It was interesting to note that, whereas the growth of rice (as manifested by increase in shoot length and dry weight) increased considerably with the increase in silt concentration from 5 to 15 per cent., no further increase in growth was observed beyond 15 per cent. concentration. At levels of 20, 25, 50, 75 and 100 per cent. silt concentration, the dry weight of seedlings and shoot length remained almost the same as in the case of 15 per cent. level. In both the cases 15 per cent. level was found to be the optimum dosage. It seems plausible that both N and K of these silt samples are directly and easily mobilized but that beyond an optimum level of concentration, no further mobilization of these elements occurs. Conforming with these results, Brouwer (1941) has mentioned that out of waste water rich in nutrients and humus, only a part of the total N and K is utilized by plants. In his comparative fertilizer experiments with waste water and pure water containing corresponding amounts of N, P and K, pure water was found superior. According to Hall (1929), N, P and K elements in sewage sludges are combined in extremely inactive form; field trials showed that the action of these sludges as manures is so small as to be negligible unless the material is applied in very large dosages. In the present investigation, however, even the application of the silt in small quantities produced marked increase in growth over that of the control; in fact the action of the silts was considerable at lower dosages of administration and no further growth response by increasing the dosage was noticed. It is, however, not clear as to why all or greater part of the N and K was not activated in larger dosages of silt application and only further work will throw light on this anomaly.

Finally it must be mentioned that these studies were undertaken essentially as preliminary experiments to a broader understanding of the fertilization and manurial values of tank silts; further experiments are being undertaken in this subject.

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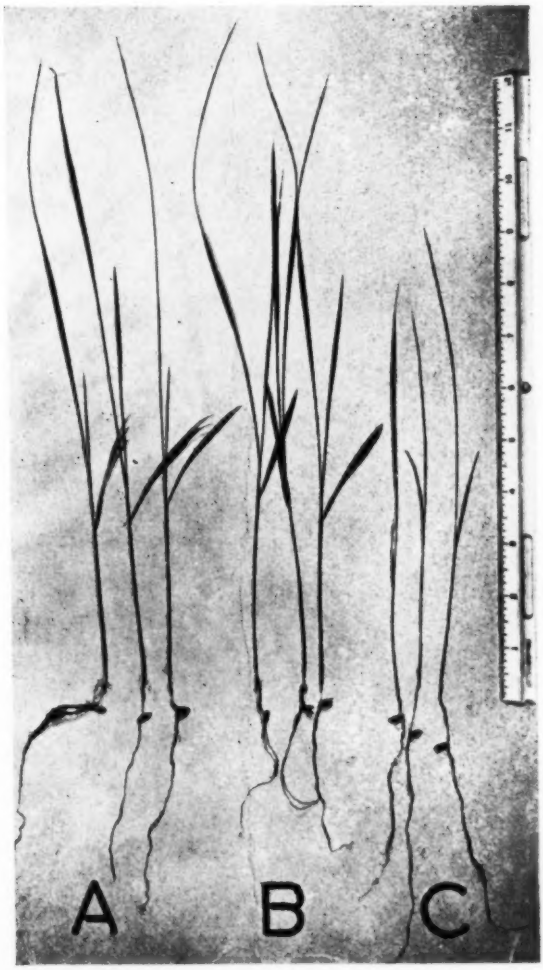

Shows the increase in shoot length of 3 weeks' old rice seedlings grown in soil with 15 per cent. silt concentration of Samples A and B over that of the control C.

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REGENERATION IN AN INDIAN EARTHWORM, *PERIONYX MILLARDI* STEPHENSON 1915

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Introduction

At one of the times when a study of regeneration in *Perionyx sansibaricus* Michaelsen 1891 was yielding only sparse and scattered data from numerous operations, living material of another species of the same genus unexpectedly came to hand. Preliminary operations on those specimens of *P. millardi* indicated that the species would provide, at least from the available source, and in comparison with specimens of *P. sansibaricus* from Allahabad, more favourable material for study of certain aspects of regeneration.

In hope of obtaining extended confirmation of some results already secured for *sansibaricus*, and additional information to fill in certain gaps, specimens of *millardi*, as they could be obtained, were also operated on.

This contribution presents a summary of results obtained from a major portion of the material provided by those operations.

Material, Methods and Nomenclature

Material was available, as far as could be discovered, only at Jubbulpore and Gaurighat. Obtaining it involved one night's travel by train each way. The collector never learned to distinguish *millardi* from *sansibaricus*, with which it was found, and the latter species provided a majority of specimens in most collections. On several occasions only a few to a very few specimens of *millardi*, in conditions to be used, were obtained. In spite of various attempts at different seasons of the year, no sexually mature individuals were ever secured, and very few specimens with any evidence of epidermal changes on clitellar segments indicative of past or future maturity. In one unusual November collection from Jubbulpore there were about 200 clitellate specimens of *sansibaricus* out of about 300 of that species. Of more than a thousand specimens of *millardi*, none were clitellate and only 24 showed a slight paling of color in the clitellar region, but spermathecal and female

pores were still unrecognizable. Some collections contained only small to medium-sized specimens. Operations were on juvenile worms, using first the larger specimens, then the medium-sized, and, when required, even submedian sizes to fill out certain series.

P. millardi has avesiculate nephridia. On specially preserved material, sites of nephropores were recognizable only with difficulty and often not certainly, or were unidentifiable even under high powers of the binocular dissecting microscope. In this respect, *P. millardi* does not provide as satisfactory material for study of regeneration as *P. sansibaricus*, for nephropores enable determination of orientation of regenerate segments as well as anteroposterior axiation of substrate fragments.

As worms became motionless in the anæsthetic they were examined under the binocular. Those with any indication of injury, disease, autotomy, amputation, or regeneration, with monstrous growths, regenerates, metameric abnormality, or homœosis of male field (other homœosis unrecognizable externally in juveniles) were discarded.

Single transections were made at levels 5/6 to 22/23 inclusive (see Table I) and at various levels from L 56 S to L 17 S. Fragments were provided by transections at: 13/14 and 30/31, 17/18 and 31/32, 30/31 and various levels from 48/49 to 119/120. In addition a considerable number of natural regenerates on substrates with single amputations were available, as well as 172 accidentally obtained fragments with regenerates at opposite ends.

At end of experiments, in some series, specimens were anæsthetized with dilute alcohol prior to preservation, a procedure that sometimes had unfortunate results.

A further statement with reference to methods will be made in contributions on regeneration in *P. sansibaricus*. Explanation of nomenclature will be found in previous contributions (see Gates, 1949 and 1950).

Circumstances arising towards the end of the last war first terminated experimentation, then study of the regenerates that had already been obtained, and finally resulted in loss of some unstudied material and records.

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TABLE I

Summary of operations providing anterior and posterior substrates, and fragments, for regeneration in Perionyx millardi

Level	Date of operation	No. of days of prior starvation	No. of operations		Time of regeneration in days		
			N	S	N	S	
5/6 ..	45-12-2	3	118	118		30	Cellar at *ca* 68-62° F. (*a*)
6/7 ..	45-12-2	3	128	128		30	do (*a*)
	45-12-6	7	100				Laboratory (at room temperature)
7/8 ..	45-10-8	8	124	124	14	14	do
8/9 ..	45-10-7	7	200	168	14	14	Half of N, all of S, in cellar at 76-78°
9/10 ..	45-10-9	9	122	122	14	14	Laboratory (at room temperature)
10/11 ..	45-10-10	10	115	115	14	44	S in cellar at 76-78° F.
11/12 ..	45-12-3	4	135	135	35	26	S and N in cellar at 68-62°F. (*a*)
12/13 ..	45-12-5	6	123	123	35	45	S and N in cellar at 68-62° (*a*)
13/14 ..	44-2-10	9	75		28		Laboratory (at room temperature)
	46-2-10	10	130				Laboratory (at room temperature)
14/15 ..	46-2-10	10	155		14-17		Laboratory (at room temperature)
15/16 ..	45-12-4	5	140	126	35	46	S in cellar at 68-62° F. (*a*)
16/17 ..	45-3-10	6	43		21		Laboratory (at room temperature)
17/18 ..	44-2-8	7	144		21		do
18/19 ..	43-10-26	7	148	148	13-21	13-21	do
19/20 ..	43-10-26	7	125	125	13-21	13-21	do
20/21 ..	44-7-31	4	115	115		21	do (*b*)
21/22 ..	44-8-1	5	119	119		21	do (*b*)
22/23 ..	44-8-2	7	124	124	12-21	21	do (*b*)
13/14-30/31	44-2-10	9	75		35		do
17/18-31/32	44-2-8	7	144		21		do
30/31—? (*c*)	44-2-10	9	75		28-35		do

LS series in this species comprised a rather small number of specimens only, of which 55 survived. Number of segments in substrates varied from 56-17. Regeneration in December-January, 1945-46, 35-50 days, 4-6 days starvation prior to operation. Additional regenerates, from accidentally provided LS substrates, also were available.

N—anterior substrates.

S—posterior substrates.

(*a*) All specimens of this series were medium-sized juveniles.

(*b*) For lack of containers all posterior substrates of three series had to be kept in one small receptacle, possibly too small.

(*c*) Substrates of 24 to 78 segments.

PART I. ANTERIOR REGENERATION BY POSTERIOR SUBSTRATES

Posterior substrate mortality following operations at 5/6 to 15/16 inclusive was zero. Mortality was higher after transection in region of 20/21-22/23 but that was probably due, at least in part, to unfavourable conditions in which the animals were kept. Effective losses were, however, higher than the mortality rate in laboratory receptacles would indicate. In

some series there were escapes from containers that could not be tightly closed. In many series some of the operated animals autotomized. In order to be certain of elimination of any influence of the autotomy itself and the condition that produced it, as well as any effect of a simultaneous, second regeneration, but at the same time to avoid discarding material secured with so much difficulty, results of regeneration by autotomized individuals were recorded but are separately presented (see Table II).

Percentage of regeneration by surviving posterior substrates was 100, in each series, including even all of the LS series. Regeneration is, however, understood in this connection to include morphologically unorganized outgrowths as well as all types of monstrous growths.

Regenerates at levels from 5/6 to 22/23 inclusive were all cephalic, with one exception, which comprised several morphologically unorganized outgrowths. Some regenerates lacked, at end of experiment, metameric differentiation, or had not yet completed such differentiation, or had metameric abnormalities of the usual types (to be considered in a later contribution). Head regenerates were also obtained, in LS series, at the following levels: L 56 S (2), L 45 S (2), L 44 S, L 40 S, L 34 S, L 33 S, L 29 S, L 26 S, L 21 S. Other substrates of unknown origin had head regenerates at L 93 S and L 65 S.

Numbers of segments in 1469 metamerically normal, cephalic regenerates of the 5/6 to 22/23 series are shown in Table II. Equimery was obtained at levels 5/6 to 12/13 inclusive, at 15/16 from one autotomized substrate only. Hypermery was first obtained at 5/6 and at all subsequent levels to 10/11 inclusive, once each at 12/13 and 15/16 but from autotomized substrates only. In each case hypermery was + 1 only. Hypomery was first obtained at 5/6 and at each subsequent level. Maximal number of segments, 17, was obtained only at 19/20 (4 regenerates) and in region of 20/21–22/23 (11 regenerates, 2 on autotomized substrates). (For explanation of eighteen and nineteen segment "regenerates" listed in Table II, see section on reorganization below.)

Numbers of segments in metamerically normal head regenerates of the LS series were as follows: at L 93 S, 14; L 65 S, 7; L 56 S, 10 and 15; L 46 S, 5; L 45 S, 8; L 44 S, 9; L 40 S, 7; L 34 S, 7; L 33 S, 7; L 26 S, 9; L 21 S, 6.

By end of thirteenth day one head regenerate, at 19/20, had completed metameric differentiation (13 segments) and was able to feed. That is a rate of segment production of one per diem for the entire period of regeneration.

Monstrous regenerates of mixed morphological nature were first obtained at L 45 S and thence from various levels back to I. 23 S. (See section on anterior monstrosities below.)

Heteromorphic regenerates were first secured at L44 S and from that level back to L 17 S inclusive. Of the 25 normal heteromorphic regenerates, 20 were metamerically undifferentiated, 18 little if any larger than an anally sculptured cicatrix, *i.e.*, comprising a narrow, perianal band of white tissue around a vertical anus centrally invaginated in the cicatrix. Number of segments in the other regenerates was: 13 at L 44 S, 2 at L 38 S (2 regenerates), 3 at L 34 S, 2 at L 22 S (anal region counted as a single segment, here large enough for two segments, one segment with complete setal circle demarcated proximally). Nephropores could not be identified on any of those regenerates nor was nephridial orientation determined.

The anus was terminal in each normal heteromorphic regenerate and in the laterally visible regenerates was slightly asymmetrical, so as to notch slightly the dorsum of the anal region. (A similar notching unrecognizable ventrally.)

Natural anterior regenerates were found at various levels from 2/3 posteriorly. Records of such regenerates will be included in a later contribution.

Evidence provided both by natural and experimental regenerates showed that anterior regenerates, sooner or later, became indistinguishable externally from their substrates.

Sex Organs in Anterior Head Regenerates

As all operations in *P. millardi* were on juvenile specimens development of sex apertures in head regenerates was not anticipated. In most regenerates no sex apertures were noticed. However, in several regenerates of the 20/21–22/23 series, spermathecal apertures were quite obvious even to casual inspection. On one fifteen-segment regenerate the pores were in 7/8, 8/9, and the left side of 9/10. On that same individual, a pair of rudimentary male pores had been developed on the second substrate segment (now xvii).

Small gonads were recognizable in some of the regenerates.

Autotomy

All autotomy in this species occurred in the first day or the first two days after operation. In fact, indication of beginning autotomy was noted in some specimens while they were still in the anæsthetic. Autotomy usually involved amputation of a single posterior portion only, the level

TABLE II

Number of segments in metamerically normal, homomorphic head regenerates on posterior substrates of Perionyx millardi

Level	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
5/6	1	4	69	4													
		2	31														
6/7		4	15	50	1												
		1	13	30	2												
7/8				21	39	2											
			1	9	32												
8/9					32	85	7										
					12	31											
9/10				2	1	24	27	2									
					2	14	16	3									
10/11						2	28	24									
					1	2	15	20	2								
11/12					1	2	35	49	16								
					2	2	5	9	6								
12/13			1	4		7	12	17	13	5							
				3		1	9	10	11	3	1						
13/14							(3)	(8)	(12)	(20)	(16)	(7)					
14/15																	
15/16									13	47	15	6					
									8	14	12	3	1	1			
16/17																	
17/18																	
							(1)	(2)	(3)	(9)	(37)	(56)	(31)				
18/19									5	13	52	64	14				
19/20										5	11	21	36	9	4		
20/21			1						1	2	3	15	56	28	9	1	
to 22/23					1		1				4	17	42	23	2	2	
Totals	1	11	131	123	126	172	154	134	75	89	98	126	149	61	15	3	

The upper row of figures, of each level, is of regenerates on unautotomized substrates. The lower row of figures is of regenerates on substrates that autotomized posteriorly at levels from 40/41 to the region of posterior end.

(All regenerates at posterior ends of anterior pieces were caudal, with but two exceptions· a cephalic monstrosity at 56/57 in the 8/9 series, an indeterminate monstrosity at 75/76 in the 9/10 series.)

Figures in parentheses, levels 13/14 and 17/18 (the only ones available for those levels), are for regenerates from fragments comprising 13/14–30/31 and 17/18–31/32 respectively. Such figures were not included in the totals.

Metameric abnormalities usually were of the same general nature as in regenerates of *P. sansibaricus*. One head regenerate, at 9/10, was marked off into seven segments, but ii, iii, v and vi each had two complete circles of setæ.

In a number of head regenerates, the buccal segment, though otherwise quite normal, was characterized by presence of one, two, several or even a number of setæ. In one regenerate of five segments (at 7/8) a complete circle was present on i. Such regenerates, if the buccal segment was of normal size, were not treated as metamerically abnormal.

in the 7/8 series from 59/60 posteriorly, in the 8/9 series from 56/57 posteriorly, in the 9/10 series from 40/41 posteriorly, in the 10/11 series from 65/66 posteriorly. However, in many cases of the series just mentioned, and in all cases of other series, autotomy involved loss of only a relatively short portion of the posterior end. In many cases autotomized posterior portions survived.

There were no cases of extensive fragmentation.

No insect larvæ, such as had often been found in autotomizing specimens of *P. excavatus* E. Perrier 1872 (see Gates, 1941, p. 167), were found at any time in any of the material of *P. millardi*. In fact, the only parasites found were external, attached to the cuticle, colonial, and with various protozoa and protophyta thereon. The ectoparasites, for which no identification could be obtained, were not involved in most cases of autotomy.

Anterior Monstrous Regenerates on Posterior Substrates

Many regenerates of the LS series, when first examined under the binocular in an early part of the regeneration period, appeared to be normally heteromorphic, usually little if any larger than an anally sculptured cicatrix, *i.e.*, with a central invagination of characteristically vertical slit-like shape into substrate gut. In numerous cases, growth of regenerate shortly began to deviate from normal. Circumstances usually did not permit continuous observation and recording of development of the monstrosities some of which soon ceased to grow. After preservation at end of period of regeneration, gross structure of monstrosities was determined. Included in a brief outline below are data from some of the more interesting cases.

Regenerate rather small, unsegmented, with terminal anus.

Regenerate normal. On dorsum of first substrate segment, which had lost all setæ and most of the pigment, a small round outgrowth nearly filled by

a spheroidal ganglion to which a branch of the nerve cord, as thick as the cord, passed. (§17)

Regenerate with right or left margin of anus enlarged and protuberant distally. Nerve cord ended abruptly without widening, thinning or flattening. (Several specimens.)

Regenerate with right and left margins of anus markedly protuberant distally, anus apparently at bottom of a terminal, vertical cleft in regenerate.

Left protuberance slight larger. Just proximal to anus, nerve cord turned to left and then up, ending in a nearly spheroidal ganglion within protuberance. (§20)

Nerve cord slightly widened and flattened proximal to anus, with a slender commissure from each anterolateral corner, left narrowing to disappearance, right passing to a spheroidal ganglion in right protuberance. A blind branch from gut passed into left protuberance. (§19)

Regenerate rather small, unsegmented, distal end more or less deeply cleft but no trace of any aperture in cleft. Nerve cord widened and flattened proximal to cleft.

Gut bifurcated, each branch into a protuberance and there with a small external aperture that was not slit-like. A commissure from each anterolateral corner of nerve cord passed into nearer protuberance, there narrowing to disappearance. (§8)

Gut, with small pharyngeal bulb, turned into left protuberance to open there by a small aperture. Both commissures from nerve cord narrowed to disappearance. (§10)

Gut bifurcated, left branch to anus on smaller protuberance, right branch with rudimentary pharyngeal bulb, opening by small aperture on right protuberance. Nerve cord passed into left protuberance. A small, slightly bilobed "brain", without recognizable commissures to nerve cord, just distal to pharyngeal bulb. (§11)

Regenerate metamerically segmented, with terminal anus. Right and left margins of anus markedly protuberant distally, anus at bottom of terminal vertical cleft. Nerve cord slightly widened and flattened proximal to anus.

With six proximal segments each with circle of setæ. Nephridial orientation? (§21)

With five proximal segments each with circle of setæ. Nephridial orientation? A commissure from right corner of distal end of nerve cord passed up to a spheroidal ganglion in right protuberance. No commissure on left side. A lateral branch of gut on each side ended blindly in protuberance. (§9)

Regenerate metamerically segmented, cephalic, normal except for a small tubercle (variously located) bearing a slit-like aperture opening through a slender tube into regenerate gut. (Several specimens.)

With six segments. Tubercle on vi just to right of mid-dorsal line. (§27)

Reorganization of Substrate Segments during Regeneration

In course of daily observations, it became obvious that changes were taking place in the first segment (part or all), or the first two segments of some substrates. Circumstances usually did not permit following such changes on individual worms but after preservation, as time was available, externally recognizable results of the process were recorded.

Homœotics having been discarded in preliminary sorting prior to operation, it was possible to recognize the segment which had been xviii in the original worm by rudiments of the male area and/or male pores. From xviii, metameres were counted forward, xviii, xvii, xvi, etc., to the level of the first substrate segment and its condition recorded. Thus, for instance, in the 11/12 series, counting forward from xviii, it was found that the segment which should have been xii of the original worm, and with a regenerate at its anterior margin, had, in 19 specimens, the same size and pigmentation as regenerate segments. The first metamere of the substrate was no longer externally recognizable as such.

Nine pairs of characteristics were found to be necessary to indicate, rather arbitrarily, externally recognizable changes that had taken place after operation. Most of the available data for *P. millardi* is summarized in Table III.

In specimens included in that table, changes apparently had taken place uniformly throughout all of the first segment but that was not always the case. In one of the 5/6 series, original pigment had disappeared from the presetal portion of vi except for clustered flecks around sites of former apertures of setal follicles. In an anterior portion of the ventrum only, an arc of new, small setæ had appeared and behind those setæ, but well in front of the equator of vi, an interesegmental furrow had been formed.

In a specimen of the 6/7 series, vii gradually bleached until the only pigment left was in rather sparsely scattered flecks in a very short circumferential band just in front of 7/8. During regeneration the size of segment vii had decreased to that of the proximal regenerate metamere.

In one specimen of the 11/12 series, xii, at the end of the experiment, was wholly unrecognizable (externally) as a substrate segment, and in addition xiii had lost all pigment and become of intermediate size.

TABLE III

Condition of first segment of posterior substrates of P. millardi *after regeneration*

Level	Time of regeneration in days	Month of regeneration	Temperatnre	Substrate size and pigment	Substrate size, intermediate pigment	Substrate size, regenerate pigment	Intermediate size, substrate pigment	Intermediate size, intermediate pigment	Intermediate size, regenerate pigment	Regenerate size, substrate pigment	Regenerate size, intermediate pigment	Regenerate size, regenerate pigment	
5/6	30	Dec.	68–62° F.					35 15	22 7				In other substrates no change in first substrate segment definitely detected
6/7	30	Dec.	68–62° F.					35 18	11 7				do
11/12	26	Dec.	68–62° F.	5	7	1	8	50	35	1	3	19	Includes autotomized as well as unautotomized substrate
12/13	45	Dec.	68–62° F.				1 3		25 12			47 (*a*) 35 (*b*)	
15/16	46	Dec.	68–62° F.				1 2	1 1	25 9			58 29	

Upper row of figures, of each level, substrates with no autotomy.

Lower row of figures, of each level, substrates from worms that had autotomized posteriorly.

(*a*) In 10 of which xiv was recognizable only by intermediate size. In six cases xiv was quite unrecognizable.

(*b*) In 7 of which xiv was recognizable only by intermediate size. In six cases xiv was quite unrecognizable.

In 17 cases of the 12/13 series (including 7 autotomized specimens) in which xiii had become wholly unrecognizable as a substrate segment, xiv also had regenerate pigment but intermediate size. In twelve further cases (including six with autotomy) xiv not only had regenerate pigmentation but also regenerate size.

In a number of cases of series 15/16, in which xvi was indistinguishable from regenerate segments, the following changes were recognizable in xvii or xvii–xviii.—Pigmentation of xvii definitely paler than of xviii but no bleaching (19, including 4 with autotomy). Segment xvii with regenerate pigmentation and intermediate size (20, including 14 with autotomy). Segments xvii–xviii with intermediate pigmentation (3). Segments xvii–xviii with regenerate pigmentation (1).

On posterior substrates with transections at levels from 18/19 back, there were, of course, no external markings, such as the male field and its pores, to enable identification of particular segments. For such substrates, condition of the first segment, or segments, definitely distinguishable externally from regenerate metameres, was recorded. In the 20/21–22/23 series. 24 specimens (including 11 with autotomy) had lost most of the pigment in the presetal half of the first segment. On 117 specimens (including 51 with autotomy) the first segment had uniformly lost some, much or nearly all of its pigment. In 14 further specimens (including 9 with autotomy) the presetal half of the second substrate segment had also uniformly lost some, much, or nearly all pigment. In nine additional specimens (including two with autotomy) the postsetal half of the second substrate segment had also been more or less bleached. In one specimen the first substrate segment had lost all pigment, most of the original setæ, and was of intermediate size.

Each " regenerate " of 18 or 19 segments (see Table II) had in the last segment, or the last two segments respectively, minute flecks of old pigment which were recognizable only under high powers of the binocular. Some regenerates of 17 or fewer segments also had similar flecks of old pigment in the proximal one or two segments.

Part II. Posterior Regeneration by Anterior Substrates

Anterior substrate mortality varied from 100% (in series at levels 5/6, 6/7, 13/14, 20/21 and 21/22) to 4% (in a series at 19/20). In the 5/6 and second 6/7 series all had died by end of fifth day. In the first 6/7 series all but one had died by end of twelfth day, the exception dead on 25th day. In a second 13/14 series there were no deaths during the first four days after operation, only seventeen during the next five, but all remaining substrates (113) died during tenth day. In the 16/17 series only one substrate died in the container (mortality in container, 2·3%), the other five that were lost having been carried off by ants. A few substrates from other series were also lost in the same way or disappeared during daily changes of cloth towelling in which specimens were kept (small substrates occasionally crawling inside hems at ends of towels). Percentages of survival at various levels are shown in Table IV.

Anterior substrates, alone of the three types used, and more especially among those of less than twenty segments, sometimes ruptured the body wall during the period of regeneration, occasionally releasing to the exterior a brownish, granular debris. No prior swelling of affected region, or other external indication of abnormality, was noticed. In the second 13/14 series

TABLE IV

Posterior Regeneration in Perionyx millardi

Level of Operation	No. of Operations	No. of Survivors	Percentage of Survival	Regenerate					
				Tail	Head	Monstrosity	Bud (*a*)	None (*b*)	Excluded
5/6 ..	118	0	0						
6/7 ..	128	0	0						
	100	0	0						
7/8 ..	124	38	30·6		27	1	3	7	
8/9 ..	200	97	48·5		72	1	8	16	
9/10 ..	122	101	82·7		70	3	16	12	
10/11 ..	115	101	87·8		69		6	26	
11/12 ..	135	80	59·2		38	13	15	14	
12/13 ..	123	67	54·4		30	19	7	11	
13/14 ..	75	1	1·3					1	
	130	0	0						
14/15 ..	155	68	43·8		31	9	8	20	
15/16 ..	140	91	65·0		52	16	15	8	
16/17 ..	43	37	86·0		31	4	1	1	
17/18 ..	144	120	83·3	1	75	25	6	8	5(*b*) (*c*)
18/19 ..	148	134	90·5	16	64	39	9	6	
19/20 ..	126	120	96·0	48	19	42	2	6	3 (*d*)
20/21 ..	115	0	0		2 (*e*)			1 (*e*)	
21/22 ..	119	0	0						
22/23 ..	124	64	51·6	39 (*f*)	7	12		6	

(*a*) With exceptions noted below, regenerates were small, of the rather conical shape characteristic of normal head regenerates at a similar stage of development, without metameric differentiation, pigmentation or terminal sculpturing. The nerve cord ended abruptly about at middle of anteroposterior length of regenerate, with no terminal narrowing as in a tail regenerate, with no broadening or flattening as in a head regenerate, and without turning laterally or laterodorsally as in some monstrous cephalic regenerates. One regenerate at 10/11, one at 15/16, and one at 17/18, was of about the same size and shape except for small monstrous protuberances. Other regenerates, 4 at 12/13, 1 at 14/15, 1 at 16/17, 5 at 17/18, and the majority of those at 18/19 and 19/20 were somewhat larger and had one or more definitely cephalic characteristics.

All bud regenerates apparently had ceased to develop some time before experiments were ended.

(*b*) See section on reorganization below.

(*c*) §1. A normal head regenerate, metameric differentiation incomplete, at 13/14.
§2. Cephalic bud, at 16/17.
§3. Monstrous regenerate of 7 segments, at 16/17.
§4. Monstrous regenerate of 8 segments, at 16/17.
§5. Segment xvii lacking, xvi regressed (see section on reorganization).

(*d*) §6. Monstrous regenerate, filament, at 14/15.
§7. Cephalic bud, at 15/16.
§8. Head regenerate of 7 segments, unpigmented, at 15/16.

Segment xvii of §5 presumably was autotomized or amputated. No clues were discovered that would indicate what happened to missing segments of other specimens.

(*e*) All specimens of original series died. Three specimens left over from another series of operations were transected at 20/21.

(*f*) In four tail regenerates at 22/23, and 26 (of 48) at 19/20, a proximal region of varying length had no metameric differentiation or only slight indications ventrally. In another regenerate a metamerically undifferentiated region was succeeded by a short region of abnormal metamerism. In 21 (of 39) tail regenerates at 22/23, metameric differentiation had been completed in the proximal region but metamerism was abnormal.

ruptures were found from fifth to tenth days and ruptured substrates (as well as all others) died. In other series ruptured specimens often survived, the site of rupture indicated at the end of experiment by a narrow cicatrix. An occasional specimen survived a second or even a third rupture, usually in vicinity of the first.

Ruptures were in almost any quadrant of any segment of subtrate from iii posteriorly, in region of junction of substrate and regenerate, or later on, entirely in regenerate. In a few cases growth of regenerate was continued, after rupture, in substrate or, more rarely, even in regenerate itself. Several head regenerates were ruined by a similar rupturing while in anæsthetic prior to preservation.

At each level back to 22/23, one or more substrates failed to regenerate. No regeneration cone or bud was observed on any substrate of the 5/6 and 6/7 series, regardless of survival time. Percentage of failure to regenerate, by surviving substrates, was higher than in posterior substrates, varying from 18·4% at 7/8 to 2·5% at 16/17.

Regeneration was first obtained at 7/8. With uniform temperature of 76° F. protrusion of cicatrix as a definitely recognizable bud was first noted on fifth day (at 8/9).

All normal regenerates at 7/8 and each subsequent level to 16/17 inclusive were heads (regenerates at 13/14 obtained indirectly, see note *c*, Table IV). Normal head regenerates were also obtained at all levels from 17/18 to 22/23 inclusive (see Table IV).

Numbers of segments in 482 metamerically normal head regenerates are shown in Table V. At each of levels 7/8 to 12/13 inclusive, and at 14/15, some regenerates had the same number of segments as the regenerating substrate. At each of levels 7/8 to 10/11 inclusive, one or more regenerates had more segments than the regenerating substrate, in each case the difference + 1 only. The largest number of segments, 15, was obtained only at evels 17/18, 18/19, and 22/23.

TABLE V

Metamerism in heteromorphic head regenerates of Perionyx millardi

Level of operation	Number of segments in metamerically normal regenerates												Metameric differentiation incomplete	Metameric abnormality	No metameric differentiation (*a*)	?	Total
	4	5	6	7	8	9	10	11	12	13	14	15					
7/8	3	7	6	5	1									2	3		27
8/9	4	10	23	14	7	1							10(*b*)	2	1		72
9/10	2	17	11	11	11	8	1						6(*b*)	3			70
10/11	1	8	14	7	17	7	4	2					5(*b*)	2	2		69
11/12		1	4	8	5	4	1	1					11(*b*)		3		38
12/13		1	1	4	6	7	1	2	1					3	4		30
13/14													1(*c*)				1
14/15				2		4	5	7	4	1	1		3			4(*d*)	31
15/16				2	7	9	9	7	6	2			6	1	3		52
16/17					1		5	8	10	5	1			1			31
17/18			1	3	4	8	12	11	18	4	3	1	10				75
18/19					1	5	2	15	14	9	3	3	12				64
19/20			1	2	1	3			1	3	1		7				19
20/21					1	1											2(*e*)
21/22																	
22/23				2			1	1			1	1				1(*f*)	7
Totals	10	44	61	60	62	57	41	54	54	24	10	5	71	14	16	5	586

(*a*) Regenerates have a normal buccal region with completely demarcated, normal prostomium but no external indication whatever of metameric differentiation.

(*b*) Proximal region of regenerate metamerically differentiated, distal region not metamerically differentiated.

(*c*) From 17/18 series. See note (*c*) under Table IV.

(*d*) Regenerate ruptured in anæsthetic.

(*e*) All of original series died. Regenerates were obtained from two of three surplus specimens of another series of operations. Regeneration on those two specimens indicates unfavourable conditions in original operations.

(*f*) Excluded from previous columns because of autotomy which took place through a region without externally recognizable evidence of metameric differentiation. In this case the regenerate was longer than any other cephalic regenerate yet obtained, in this or other species. The distal extremity was sculptured to form a buccal region that appeared normal. Some metameric differentiation was recognizable on a relatively short distal portion. A proximal region remained unpigmented, without externally recognizable evidence of metameric differentiation, and continued to grow through nearly all of the regeneration period. Eventually two annular constrictions appeared in the unpigmented region. During the 20th day deepening of the more distal constriction ended in autotomy. Both pieces survived and healed over. No further growth or differentiation was observed. The autotomized portion comprised a normal head of six segments and a short, metamerically undifferentiated region.

Monstrous regenerates at levels 7/8 to 16/17 inclusive were either morphologically undetermined (for head or tail) or were cephalic only (see Table VI). Monstrosities of mixed morphological nature were first obtained at 17/18.

Tail regenerates were first obtained at 17/18 (one of 120 surviving specimens with normal tail, one with caudal monstrosity), and normal tails were secured at each subsequent level to 22/23 inclusive. Percentages of tails among normal regenerates: at 17/18, 1·3%; 18/19, 20%; 19/20, 71·6%; 22/23, 84·7%.

The normal tail regenerate at 17/18, at end of 21st day (February), had a length of 6 mm. (substrate length, 7 mm.) and 34 setigerous segments; rate of segment production, 1·6 + per diem for entire period. However, the proximal portion of regenerate was still metamerically undifferentiated dorsally though ventrally marked off into three setigerous segments, and proximal to anal region there were indications of still other segments on which setæ were unrecognizable.

Natural tail regenerates, at many posterior levels, were obtained in most collections but will be considered elsewhere.

No evidence was found, either in naturally or experimentally produced tails, that a caudal regenerate ever becomes indistinguishable from its substrate.

Autotomy

No autotomized fragments, or stains on towelling that might indicate decay of autotomized fragments, were observed in case of anterior substrates. Nevertheless one substrate (at least) must have undergone some sort of an amputation resulting in loss of one segment (see §5, Note *c*, Table IV).

One case only of autotomy through a regenerate was observed (see Note *f*, Table V).

Posterior Monstrous Regenerates on Anterior Substrates

Monstrous posterior regenerates were more diverse than those on posterior substrates (see Table VI). Morphologically indeterminate regenerates were of the same types as had been obtained in *P. sansibaricus* and which are to be considered in a later contribution. Some of the more

TABLE VI

Monstrous posterior regenerates of anterior substrates in Perionyx millardi

Level of operation	Monstrosity morphologically determined			Monstrosity indeterminate			
	Caudal	Cephalo-caudal	Cephalic	Filament (*a*)	Dome (*a*)	Misc.	Total
7/8 ..				1			1
8/9 ..						1	1
9/10 ..				2		1	3
10/11 ..							
11/12 ..			2	3	1	7(*b*)	13
12/13 ..			1	10	2	6(*b*)	19
13/14 ..							
14/15 ..			1	1		7(*b*)	9
15/16 ..				3		13(*b*)	16
16/17 ..			3			1	4
17/18 ..	1	1	9	8	3	3	25
18/19 ..	4	2	24	5	3	1	39
19/20 ..							42
20/21 ..							
21/22 ..							
22/23 ..	1		6			5	12
Totals ..	6	3	46	33	9	45	142+42

(*a*) These types of monstrous regenerates will be considered in a later contribution on regeneration in *P. sansibaricus*.

(*b*) Monstrous condition of regenerate in part, or entirely, result of earlier, terminal or subterminal rupture in regenerate.

interesting, morphologically determined monstrosities are briefly characterized below.

Monstrosity cephalic.

Regenerate metamerically segmented and with normal buccal region and prostomium.

Regenerate, of 12 segments, with a small, ventrally directed, white growth from mesial portions of x–xii. Nerve cord widened and flattened in x–xii, perforated by a slender tube that passed straight down from regenerate gut to open to exterior by a minute round aperture at distal end of outgrowth.

Regenerate, of 8 segments, at 17/18, with ventral outgrowths on i and vii. Regenerate, at 11/12, with outgrowth near substrate.

Regenerate metamerically differentiated, number of segments varying from 5 to 12. Distal portion (counted as one segment) rather conical, with no setæ, buccal, prostomial or other sculpturing. With a pharyngeal bulb in blindly ending regenerate gut and/or with some definitely cephalic characteristics in regenerate nervous system.

Seven regenerates at 17/18, others at 14/15 and 16/17.

On iii, just left of mid-dorsal line, a small papilla with a slit-shaped aperture which opened through a slender tube into regenerate gut. Brain and commissures present. Regenerate with 12 segments. (Cephalocaudal?)

Monstrosity cephalocaudal.

A short proximal region metamerically differentiated and with distal nephropores (heteromorphic orientation). A middle region, metamerically undifferentiated, or with rudimentary intersegmental furrows, or, rarely, metamerically differentiated but nephridial orientation indeterminable. Distal region metamerically differentiated and normally caudal. Regenerates at 18/19 and 19/20.

Distal end dorsoventrally bifid. Dorsal bifurcation a buccal segment (without setæ) and with normal prostomium. Ventral bifurcation a short tail. Regenerate gut bifid, dorsal branch with pharyngeal bulb, ventral branch with none. Nerve cord continued beyond commissures, which pass up to a brain, into tail. (At 19/20.)

Monstrosity caudal.

Anus unusually large, with irregular margin, on right, left or ventral sides. In one, a ventral anus wholly proximal to distal end of regenerate. (1 at 17/18, several at 19/20.)

Monstrosity developed subsequent to rupture through regenerate. Some distance distal to rupture regenerate was bent to right and then proximally, into a U-shape with tip overlapping substrate. Metameric differentiation only on outer sides of limbs which were united distally.

Reorganization of Substrate Segments during Regeneration

In one specimen of the 7/8 series, setæ of vii disappeared and the whole segment, as well as the cicatrix at 7/8, grew smaller though without recognizable change in parietal pigmentation. At end of experiment (14 days) vii was represented only by a flat, circular band of normal substrate pigmentation around a white cicatrix, band and cicatrix constituting a circular marking, without recognizable segmental thickness, on posterior face of vi.

In a substrate of the 9/10 series, ix and cicatrix at 9/10 were similarly reduced to a circular marking on posterior face of viii. In one specimen of the 17/18 series, presumably after amputation of xvii, xvi and cicatrix at 16/17 were reduced to a circular marking on posterior face of xv. In another specimen from the 17/18 series, setæ disappeared only from the dorsal half of xvii which was then reduced to a flat marking of normal parietal pigmentation on posterior face of xvi and upper surface of apparently unmodified ventral half of xvii. (A slender, white fllament even grew out, from a ventral portion of cicatrix that was in contact with substrate nerve cord, during period while dorsal regression was under way.)

In a substrate of the 7/8 series, vii not only lost most of its pigment and all of its setæ but even acquired the rather conical shape characteristic of a cephalic bud. Except for presence of minute flecks of substrate pigment, recognizable only under the binocular, the cone would have appeared to be a regenerate at 6/7.

In those specimens of series 17/18 with most rapidly developed regenerates, little or no change was recognized in the last (proximal) substrate segment. In other specimens a postsetal, circumferential portion of xvii, of variable length, was slightly or almost completely bleached, in the latter case sometimes with one to several quite small, irregular areas of unchanged pigmentation. In a few cases, apparently without change in substrate pigmentation, one or two incomplete circles of new, small setæ had been developed in postsetal portion of xvii. In one such case a rudimentary intersegmental furrow, with rudimentary dorsal pore, was recognizable between the two new setal arcs. In one substrate the dorsal half of xvii was bleached, the ventral half apparently unchanged. In another specimen, flecks of substrate pigment showed that xvii had been reorganised into two (or more?) segments of the proximal part of a monstrous regenerate.

In most specimens of the 22/23 series with tail regenerates, little or no change was recognized in the last substrate segment. In other specimens xxii was definitely shortened, in two cases being represented only by a major part of the presetal portion. No substrate-pigment flecks were recognizable in the proximal segments of regenerates. The latter were sharply demarcated from substrates by normal intersegmental furrows with functional dorsal pores therein.

Part III. Regeneration from Simultaneous Anterior and Posterior Transections

Fragment mortality varied from 1·4% to 28%. Three sets of 75 substrates each (44-2-10), anterior substrates of 13 segments, fragments of 17

segments (13/14–30/31), fragments of various segment numbers (30/31–?), were from the same 75 worms. Percentages of survival in those three sets were: 1·3%, 92%, and 72% respectively. Two sets of 144 substrates each (44–2–8), anterior substrates of 17 segments, fragments of 14 segments (17/18–31/32), were from the same 144 worms. Percentages of survival were 83·3 and 98·6% respectively (actually but 17 of the lost 24 anterior substrates died in containers).

In the 44–2–10 series, every surviving fragment regenerated at the anterior transection and all but five regenerated at the posterior transection also. The sole surviving anterior substrate of the same series of worms did not regenerate. In the 44–2–8 series, every one of 142 surviving fragments regenerated anteriorly and only two failed to regenerate posteriorly. Eight of 120 surviving anterior substrates of the same series of worms failed to regenerate.

In all four series of fragments, a majority of anterior regenerates were cephalic. In the first two series a majority of normal posterior regenerates were also cephalic (heteromorphic). In the third series, from a more posterior region, a majority of normal posterior regenerates were caudal (homomorphic). The types of regenerates obtained in the four series of fragments are shown in Table VII.

Numbers of segments in head regenerates of the first two series, regardless of nature of posterior regenerate, are shown (in parentheses) in Table II. Numbers of segments in metamerically normal head regenerates of third and fourth series respectively were: 10 (5 specimens), 11 (2), 12 (5), 13 (11), 14 (13), 15 (11); 6 (2), 7 (2), 8 (2), 9 (2), 10 (2), 11 (4), 12 (2), 13 (9), 14 (32), 15 (57), 16 (27), 17 (5), 18 (2), 19 (1).

Head regenerates at both transections.—Substrates, in the third and fourth series, comprised 37–58 and 6–33 segments respectively (but see note *b*, Table VII). Levels of heteromorphic head regeneratior, known only in the third series, ranged from 67/68 to 88/89. However, possibility of normal head regeneration at the level next behind was indicated by a well-developed cephalic monstrosity (of 6 segments) at 89/90.

The posterior head was always distinguishable from the anterior by smaller size (length usually shorter, diameter less), less well-developed pigmentation, and usually by a smaller number of segments.

In substrates of 6, 7 and 8 segments, combined lengths of the two head regenerates were greater than the length of the substrate. In a six-segment fragment, the total number of metameres in the two heads was 26, more

TABLE VII

Nature of regenerates on fragments of Perionyx millardi

Anterior regenerate	Posterior regenerate	Series 1 13/14/–30/31	Series 2 17/18/–31/32	Series 3 30/31–?	Series 4 Levels unknown
Normal head	Normal head	26	112	5	30
	Cephalic bud (*a*)	1	..	..	..
	Cephalic monstrosity	22	25	8	1
	Cephalocaudal monstrosity	1	..	..	..
	Caudal monstrosity	1	..	..	..
	Normal tail	1	1	38	136
	Indeterminate monstrosity	10	..	..	..
	No posterior regenerate	5	2	1	..
Cephalic monstrosity	Cephalic bud (*a*)	1	1	..	..
	Cephalic monstrosity	1	..	..	..
	Normal tail	..	..	2	..
Normal tail	Normal tail	..	..	..	6
None ..	Normal tail	..	..	..	1
Excluded above		..	1(*b*)	..	..

(*a*) These regenerates had no definitely cephalic structures, the shape of the bud that of a normal cephalic regenerate at a corresponding stage of development.

(*b*) Shortly after regenerate buds were recognizable the substrate broke into two unequal portions. The larger was lost. The smaller, of five segments, at termination of experiment, had two metamerically normal head regenerates, the anterior with 12 segments, the posterior with 7 segments.

than four times that in the substrate. In the excluded five-segment fragment of the 17/18–31/32 series, total number of metameres in the two heads was 19, nearly four times the number in the substrate. All substrates of 25 and more segments had more metameres than in the two regenerates combined.

Numbers of segments in both regenerates are shown in Table VIII. Number of segments in heteromorphic head regenerates of the 30/31–? series was: at 67/68, 4 segments; at 70/71, 13; at 75/76, 6; at 80/81, metamerically abnormal; at 88/89, 8.

Head regenerate at anterior transection, tail at posterior.—Number of segments in substrates (third and fourth series) varied from 6 to 194. The longest tail regenerate was in the 30/31–? series, and at 84/85. It was 14·5 mm. long and comprised 34 setigerous segments and a large growth zone. Thus the substrate, of 34 segments, had produced, in four weeks, new segments in the tail alone at a rate of 1·2 + per diem for the entire period.

TABLE VIII

Number of segments in regenerates on fragments of Perionyx millardi *with two metamerically normal head regenerates*

Anterior head	Posterior head	No. of specimens of substrates comprising			Totals
		13/14–30/31	17/18–31–32	30/31–?	
9	9	1	..	..	1
10	9	..	1	..	1
	10	3	1	..	4
11	8	2	..	..	2
	9	1	..	..	1
	11	..	2	..	2
12	8	1	..	..	1
	9	1	1	..	2
	10	3	1	..	4
	11	..	4	..	4
	12	3	2	..	5
13	6	..	..	1	1
	7	1	..	..	1
	8	..	1	..	1
	9	..	3	..	3
	10	2	3	..	5
	11	1	6	..	7
	12	2	13	..	15
	13	1	2	1	4
14	8	..	..	1	1
	9	..	4	..	4
	10	1	5	..	6
	11	1	9	..	10
	12	..	14	..	14
	13	2	10	..	12
	14	..	2	..	2
15	4	..	..	1	1
	8	..	2	..	2
	9	..	1	..	1
	10	..	3	..	3
	11	..	3	..	3
	12	..	6	..	6
	13	..	7	..	7
	14	..	5	..	5
	15	..	1	..	1
	Totals ..	26	112	4	142

The smallest heteromorphic head obtained in *P. millardi* comprised two segments. Both brain and pharyngeal bulb were small and in ii.

In addition, during the same period, a normal head of 15 segments had been formed.

Tail regenerates at both transections.—Number of segments in substrates (all from fourth series) ranged from 9 to 23. All substrates were almost certainly from a region behind 40/41 and size of substrate metameres indicated origin from a region near posterior end. All tail regenerates, with but one exception, were not metamerically segmented. The exceptional regenerate, heteromorphic and *at anterior transection* of a 13-segment substrate, had 5 setigerous segments and a growth zone. The regenerate was unpigmented and nephridial orientation was indeterminable.

The anus in each regenerate was terminal.

Monstrous Regenerates on Fragments

Monstrosity not morphologically determined.

Regenerate gradually widened distally. Unpigmented. No metamerism though long enough for several segments. Outer wall richly vascularized and similar in appearance to gut wall. A rather large, atypical aperture into gut terminally. (Body wall and gut wall healed together? Regenerate produced by growth of gut?) 2 posterior regenerates at 30/31.

Monstrosity morphologically determined.

Cephalic.

A. Regenerate metamerically differentiated (one exception) but usually unpigmented. Nephridial orientation cephalic. Distal segment rather conical, without setæ and sculpturing (occasionally second segment also lacking setæ). Number of segments, 3–13; 0 (1 regenerate), 3 (1), 4 (2), 5 (4), 6 (4), 7 (6), 8 (5), 9 (6), 10 (6), 11 (3), 12 (6), 13 (2). Gut with blind distal end; without marked change in thickness terminally, or narrowed distally to a slender filament, or widened subdistally to a thin-walled vesicle (in iii or iv) and with or without a much slenderer distal continuation. Vascular system cephalic, with hearts. 46 + posterior regenerates.

No brain. No pharyngeal bulb. Nerve cord ending abruptly in i, ii, or iii. 8 + posterior regenerates. In a posterior regenerate at 73/74 gut continued to end of regenerate. Nerve cord passed up on right side and to end of regenerate, ending abruptly (§§ 47–54).

No brain. Pharyngeal bulb present. Nerve cord ending abruptly in iii, or slightly flattened and widened under pharynx, or continued to distal end of regenerate with little or no diminution or with slight and gradual narrowing. Several posterior regenerates. In one regenerate the nerve cord at first appeared to end under pharynx but actually was continued by a slender filament to distal end of regenerate (§§61).

Brain present. No pharyngeal bulb

Nerve cord ending abruptly in iii or iv.

A spheroidal ganglion (brain) dorsally in ii or iii. 2 posterior regenerates (§§70–71).

A bilobed brain present. In one case brain small but bulging inwards anterior wall of gut vesicle. In another regenerate (with no metamerism) much larger but not indenting gut vesicle (§§75–76).

Nerve cord ending in a spheroidal ganglion. Several regenerates (§78).

Brain present. Pharyngeal bulb present. 8 + posterior regenerates.

Nerve cord ending abruptly in iii. Brain spheroidal in ii, distal to blind end of gut. 1 regenerate (§83).

Nerve cord ending abruptly in iv. A small, bilobed brain dorsally on pharynx, in ii or iii. 2 regenerates (§85–86).

Nerve cord, distal to gut, ending in a spheroidal brain.

Ganglion imperforate. 1 regenerate (§87).

Ganglion perforated by a slender filament from gut, apparently not continued distally. 1 regenerate. (§88).

Ganglion perforated by a narrow but tubular continuation of regenerate gut that passed to distal end of regenerate. 1 regenerate (§89).

B. As in A but distal segment (asetal) bluntly rounded. Surface slightly roughened terminally but otherwise unsculptured. A bilobed brain rested on pharynx which was in contact with distal end of regenerate. 1 anterior and 1 posterior regenerate (§92).

C. As in A but with small knob-like protuberance on iv just to right of mid-dorsal line. Gut ending behind brain. A dorsal branch from gut opening by anal-like slit on tubercle. (Cephalocaudal? Induction of heteromorphosis in homomorphic tail?) 1 posterior regenerate at 31/32 (§93).

D. As in A but at tip of conical, distal segment a very small, blind (?) invagination. Brain in iii, perforated by a slender filament from gut vesicle that was continued to distal end of regenerate. 2 posterior regenerates, at 31/32 (§94–95).

E. As in A but terminal segment, rather conical, continued distally as a slender, unsegmented, threadlike protuberance. No pharyngeal bulb. No brain. Nerve cord continued into base of protuberance and there very slightly thickened. 1 posterior regenerate (§96)

F. Regenerate metamerically segmented. Nephridial orientation cephalic. Distal end with small, right and left buccal regions, each with prostomium. Gut bifurcated, distal to pharynx, each branch to a mouth. Nerve cord passed to left side and above gut branch to distal end of regenerate there terminating abruptly. No brain found. Posterior regenerate at 30/31 (§22).

Cephalocaudal.

Distal end conical, unsculptured. Gut turns to side to open by a vertical anus. Nerve cord continued to spheroidal brain at distal end of regenerate (Nephridial orientation?)

Regenerate unsegmented (rudiments of several intersegmental furrows indicated?) Anus on left side about half way toward distal end of regenerate. 1 posterior regenerate at 30/31 (§37).

Regenerate segmented. Anus on right side. 1 posterior regenerate at 85/86 (§38).

Reorganization of Substrate Segments during Regeneration

In the first series, one substrate segment had become completely unrecognizable as such, in 8 fragments (of 69 surviving). In two others (with no posterior regenerate), xxxi had been reduced to a rudimentary circular area on the posterior face of the penultimate metamere. In the second series, one substrate segment had become completely unrecognizable as such, in 35 fragments (of 142 surviving), while two substrate segments had become unrecognizable as such in 8 other fragments. In seven specimens of the second series, persistent minute flecks of old substrate pigment indicated origin from xxxi of 2 (5 specimens) or 3 (2 specimens) proximal segments of posterior regenerate.

In 62 of the first series and 88 of the second series, the first recognizable substrate segment was of intermediate size and with regenerate pigmentation. The body wall was slightly thinner than in the next substrate segment, nephridia were recognizably smaller, and the gut was somewhat narrowed and in macroscopic appearance more like that of regenerate gut.

The first recognizable substrate segment of certain other fragments was characterized as follows:—Intermediate in size; with substrate pigmentation (1), or with regenerate pigmentation in anterior half, substrate pigmentation in posterior half (1), or considerably blanched (1), or blanched except for a short strip by posterior intersegmental furrow (1), or completely blanched (2). Of regenerate size but with substrate pigmentation (1). First two substrate segments of intermediate size and with regenerate pigmentation (1).

Similar conditions characterized the last substrate segment of a few fragments. Last substrate segment completely blanched except for a band of unchanged pigmentation behind anterior intersegmental furrow (1). Posterior half of last substrate segment almost completely blanched (1). Last substrate segment completely blanched and without setæ, sites of nephropores unrecognizable (1).

Usually reorganization in the last substrate segment had involved no recognizable decrease in size, little or no loss of setæ, no recognizable loss of pigment. Nevertheless, without any such changes, new structures had been developed in the last substrate segment of 114 specimens of the first two series. These new structures included new and smaller setæ, in a short transverse row, or in a semicircle, or in a nearly complete circle, in posterior half of last substrate segment. Occasionally, in front of the new setæ but behind the old, there was recognizable a rudimentary intersegmental furrow (or part of one), sometimes containing a rudiment of a dorsal pore. Behind that furrow, in at least 15 specimens, a new pair of small nephropores had become visible. Corresponding to those pores, there was internally a pair of new and small nephridia.

Discussion

For the first time, for any species of earthworm, there have now been published, records of regeneration, at single transections, at exact levels, in both directions, from numerous operations, in the very important anterior portion of the axis. Also recorded for the first time are results of regeneration, again in both directions, at various levels, in an important posterior portion of the axis.

Anterior regeneration was obtained from posterior substrates of *P. millardi*, at various levels from 2/3 back to L 17 S. No evidence is available, either from work on this or any other species of earthworm, to indicate inability to regenerate anteriorly in a limited region (in this case, in front of 2/3) or at various intermediate levels between those for which records are available. Undoubtedly, in *P. millardi*, anterior regeneration is possible at all levels back to L 17 S at least, with no zero level anteriorly. Whether or not posterior substrates of 16 or fewer segments can regenerate remains to be determined.

Posterior regeneration by anterior substrates was obtained experimentally at all levels from 7/8 to 22/23 inclusive, and was found naturally at various levels back to 204/205, with no indication of zero level posteriorly. There is no reason to suspect inability to regenerate at intermediate levels between

those for which records are available. Undoubtedly, in *P. millardi*, posterior regeneration is possible from 7/8 back, with no zero level posteriorly.

In an anterior region extending back to 16/17 inclusive, all morphologically determined regenerates were cephalic. That region accordingly appears to be one of unipotential regenerative capacity only. From 7/8 to 16/17 inclusive, regenerates were obtained at both anterior and posterior transections (of posterior and anterior substrates). In that part of the anterior, unipotential region there obviously is no directional restriction of regenerative capacity. In a small anterior part, from 6/7 inclusive forwards, regenerates were obtained from anterior transections only. That is suggestive of a directional restriction on regeneration. However, anterior substrates of six and five segments have not yet been kept alive, in numbers, long enough to demonstrate species inability to regenerate. Fragments of five- and six segments, from other regions of the body, not only regenerate but even do so rapidly and normally.

In the region from 17/18 to 22/23 all anterior regenerates were cephalic but posterior regenerates at each level were cephalic, or caudal, or even of a mixed morphological nature, *i.e.*, with both cephalic and caudal characteristics simultaneously. Accordingly, in the region of 17/18 to 22/23 inclusive, regenerative capacity may be said to be bipotential. Nevertheless the bipotentiality appears to be limited by direction of regeneration at transections at anterior end of substrate but not at the same levels when at posterior end of substrate.

The percentage of heteromorphic heads among normal regenerates at posterior transections decreased from 100% at 16/17 (98·5% at 17/18, 80% at 18/19, 28·3% at 19/20) to 15·2% at 22/23, the last level of operation. The posterior limit of heteromorphism, accordingly, has not been determined, but presumably may be expected to be in the region of 27/28.

Behind the limit of posterior heteromorphism, for some distance, only cephalic regenerates are to be expected at anterior transections, and only caudal regenerates at posterior transections. This region is one of complete directional limitation of bipotentiality at both anterior and posterior transections. This region will be bounded posteriorly by the anteriormost level of anterior heteromorphism. That boundary, at present, can only be tentatively placed, and with reference to the posterior end, at L 44 S. As the number of segments in *P. millardi* may be as many as 220 the boundary for a long individual can be at least as far back as 176/177.

Anterior heteromorphism was obtained at various levels from L 44 S to L 17 S inclusive. Undoubtedly at each of the intermediate levels, for which no records are yet available, heteromorphosis is also possible. At various levels from L 44 S to L 21 S inclusive, head regenerates were obtained anteriorly. There is no reason to suspect inability to regenerate heads at any of the intermediate levels. In this posterior region, posterior regeneration can be assumed to be caudal. Accordingly, in a region extending from L 44 S to L 21 S inclusive, the bipotential regenerative capacity is without directional limitation at anterior transections but is directionally limited at the same transections when posterior. This is the reverse of the condition apparently characteristic of the region from 17/18 to 22/23 (→27/28 ?).

At levels L 20 S to L 17 S inclusive, only caudal regenerates were obtained at anterior transections. Here again posterior regenerates can be assumed to be caudal. Accordingly, this region has a unipotential regenerative capacity only. The posterior limit of anterior heteromorphism has not been determined. Presumably there is such a limit and consequently a directional limitation on regenerative capacity the reverse of that apparently characterizing the anterior unipotential region.

The pattern of regenerative capacity as thus outlined is represented diagrammatically in Fig. 1. It is essential to bear in mind that boundaries of various regions are, as indicated above, in part tentative and subject to future revision. Also worthy of special note is the fact that the pattern shown in the diagram, as well as all of the preceding portion of this discussion, is based only on regeneration from anterior and posterior substrates, *i.e.*, substrates having a single transection at which to regenerate.

The number of morphogenetic regions in *P. millardi*, as outlined above, is the same as that recently proposed (Gates, 1949, 1950) for the Lumbricid species, *Eisenia fœtida* (Savigny), 1826. Definite demonstration of an anterior region of unipotential regenerative capacity, with nearly the same limit, and with similar subregions or sections, in *P. millardi*, provides some additional, though indirect, support for conclusions reached in the study of *E. fœtida*.

The anterior boundary of the region of bipotential capacity in *E. fœtida* was placed at 18/19 by two of a series of 33 regenerates. The corresponding boundary in *P. millardi* was placed at 17/18 by three out of 107 regenerates from 144 operations at that level. In *E. fœtida*, the region of bipotential capacity appears, at present, to differ from that of *millardi*, by absence in a large portion, of any directional restriction of the morphogenetic bipotentiality.

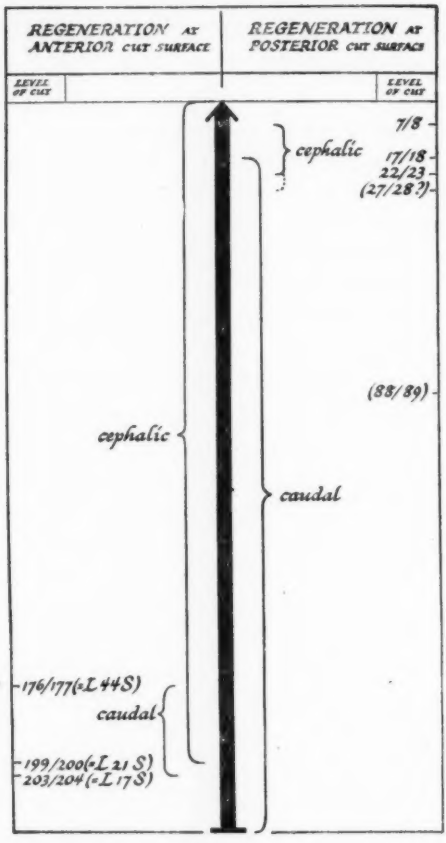

FIG. 1

Regeneration along main axis of *Perionyx millardi* after one transection. (Heteromorphic head regeneration possible back to 88/89 on fragments with two simultaneous transections.)

The posterior region of unipotential capacity of *E. fœtida* appears at present to be very much larger than in *P. millardi*.

In a previous contribution (Gates, 1950, p. 43), it was suggested, on a basis of rather slight evidence, that earthworms might have the ability to produce "as many segments in heteromorphic as in homomorphic head regenerates" at the same level. Further supporting evidence is provided in Table IX.

The pattern of regenerative capacity of *P. millardi*, as outlined above is quite incomplete, having reference only to substrates with single transec-

TABLE IX

Number of segments in homomorphic and heteromorphic heads of P. millardi

Level	Homomorphic	Heteromorphic
7/8	6–8	4–8
8/9	7–9	4–9
9/10	6–10	4–10
10/11	8–10 (*a*)	4–11
11/12	7–11	5–11
12/13	5–12	5–12
13/14	(9–14) (*b*)	
14/15		7–14
15/16	11–14 (*c*)	7–13
16/17		8–14
17/18	(9–15) (*b*)	6–15
18/19	11–15	8–15
19/20	12–17	6–14
20/21	5–17 (*d*)	
21/22	5–17 (*d*)	
22/23	5–17 (*d*)	7–15

(*a*) 7–11 on autotomized pieces.

(*b*) On fragments, see Part iii.

(*c*) 11–16 on autotomized pieces.

(*d*) No data for individual levels. Excluding 18- and 19-segment regenerates with last two segments reorganized from substrate.

tions. Regeneration may take place on a fragment with two transections which can be made simultaneously or at different times. Such regeneration by fragments must be considered if more complete characterization of pattern is to be secured.

None, or at most very few, heteromorphic head regenerates can be expected on anterior substrates from posterior transections at 30/31. At 31/32 even fewer heteromorphic heads can be expected. Yet, in two series of fragments comprising segments xiv–xxx and xviii–xxxi, 26 of 27 and 112 of 113 fully developed, normal, posterior regenerates respectively, were heteromorphic. Obviously another transection, or a regeneration taking place simultaneously at that second transection, in certain circumstances, can influence the morphological nature of the regenerate developed at the other transection. This induction of heteromorphosis should not be confused with the induction of homomorphosis (see below) which takes place at a later period.

The four series of Part III clearly show that in fragment regeneration two factors have to be taken into consideration, number of segments in the

regenerating fragment, and position of the fragment on the anteroposterior axis. Only one experiment was designed to throw light on such a complicated situation but the data derived therefrom are too slight to warrant further discussion at present.

It is, however, noteworthy, that a heteromorphic head was obtained, on a fragment, at level 88/89 and that a cephalic monstrosity indicated possibility of heteromorphism at 89/90. This is about 60 levels behind the limit of posterior heteromorphism for anterior substrates. Yet there is little evidence to indicate that the limit of posterior heteromorphism for fragments has been found, in *P. millardi.*

The problem of characterizing pattern of regenerative capacity in *P. millardi* is rendered still more difficult by several types of " regulation ", which with but one exception have not been recorded hitherto from earthworms, nor, so far as can be discovered from a hasty search through the literature, from other metamerically segmented animals.

In cases of one of those types, the regenerate, during growth appeared to be normally heteromorphic. However, after preservation, microscopic examination showed that segments in a proximal region of the regenerate had nephridia oriented as in substrate rather than as in the rest of the regenerate. Thus, an apparently normal heteromorphic head had proximal segments with nephridial orientation of a tail regenerate and a heteromorphic tail had proximal segments with nephridial orientation of a head regenerate. (Anteroposterior cephalocaudal monstrosities.)

Another type of regulation (induction of homomorphosis) involved heteromorphic tail regenerates and subsequent monstrous growth therein. The end result, however, of the process, in certain successful cases, was an almost normal head regenerate (as in §27, anterior monstrous regenerates on posterior substrates). In unsuccessful cases growth had ceased at various times prior to preservation thus providing interesting intermediate stages (see sections on monstrosities). Induction of heteromorphosis (incomplete) in a homomorphic regenerate seems to be indicated by two specimens (§37–38).

Yet another type of regulation, this time within substrate, still further complicates the regenerative pattern of *P. millardi.* In some cases the reorganization is similar to that already reported from *P. excavatus* E. Perrier 1872 in a previous contribution (Gates, 1941). In both species, the reorganization was definitely demonstrated by persistence, in occasional specimens to time of preservation, of scattered, minute flecks of original substrate pigment in segments that appeared, except under the binocular, to be indis-

tinguishable from metameres of the regenerate proper. In *P. millardi*, reorganization of substrate segments usually was recognized only after some growth of the regenerate. In special circumstances, however, reorganization of substrate segments became quite obvious some time before any protrusion of the cicatrix was detected. Another method of reorganization, in *P. millardi*, seems to be indicated by the appearance of arcs of setal circles and intersegmental furrows (sometimes with rudiments of dorsal pores) in substrate segments which apparently had lost no pigment. Reorganization of a substrate segment may even be able to produce a structure with characteristics of a regenerate at a level where regeneration proper does not take place (see Part II, reorganization, by a seven-segment anterior substrate, of vii into a structure with external appearance of a cephalic bud regenerate).

All such types of reorganization, closely associated with regeneration, can be regarded as of a constructive or progressive nature. In absence of regeneration, reorganization, presumably of a somewhat different nature, may result in reduction of one or more substrate segments to obviously useless vestiges. This sort of reorganization, which may be called destructive or regressive, apparently has not hitherto been recorded from earthworms.

An organizing role of considerable importance in head regeneration has been attributed, at times, to the earthworm brain. Indeed, "The establishment of a brain anlage is always the first event in head regeneration," according to Painter (1940, p. 478).

In the single specimen of *P. millardi*, in which a "brain" was found in the substrate, that brain was associated with an abnormal outgrowth at some distance from the level of transection and regeneration.

In each specimen of *P. millardi* with a bud (inhibited) regenerate, that had the characteristic conical shape and appearance of a normal head regenerate of the same size, no brain was found in either regenerate or substrate.

In numerous, monstrous, cephalic regenerates characterized on previous pages, the following cephalic characteristics, always in apparent absence of any brain, were noted: characteristic subœsophageal ganglia in nerve cord, partial development of circumœsophageal commissures; cephalically organized gut, including buccal cavity, œsophagus and pharynx; cephalically organized circulatory system with "hearts"; nephridia in cephalic orientation; normal buccal region and prostomium. Even in a distally bifurcated regenerate with two buccal regions (each with prostomium)

no brain was found. In such regenerates there were no caudal characteristics whatever, and number of segments was sometimes as many as thirteen.

Since regenerates may have, collectively, all other characteristics and structures of a normal head, in apparent absence of any structure recognizable as a brain, it would seem that in *P. millardi* original determination of morphological nature of the head regenerate can be independent of the presence of a brain.

Summary

Regeneration by anterior and posterior substrates (having a single transection each), indicated presence along anteroposterior axis of three morphogenetic regions. (1) A region of unipotential capacity from 16/17 anteriorly, in which all morphologically determined regenerates were cephalic, with a section from 6/7 anteriorly in which regeneration was obtained only anteriorly. (2) A region of bipotential capacity in which morphologically determined regenerates were cephalic or caudal. Apparent directional limitations of bipotentiality enabled recognition of three sections: 17/18 to 22/23 (→ 27/28 ?) in which regenerates at anterior transections were all cephalic but at posterior transections were cephalic or caudal or cephalocaudal; a long middle section in which all anterior regenerates were cephalic and all posterior regenerates were caudal; a posterior section in which regenerates at anterior transections were cephalic or caudal or cephalocaudal but at posterior transections were all caudal, tentatively bounded by 176/177 and 200/201. (3) A region of unipotential capacity in which all morphologically determined regenerates were caudal. Experiments were not extended to permit recognition of a terminal section in which regeneration would only take place posteriorly.

The pattern of regenerative capacity thus indicated is complicated, in *P. millardi*, by induction of posterior heteromorphosis by a simultaneous anterior regeneration. with heteromorphic cephalic regeneration possible at least as far back as 88/89 and by various types of regulation. Induction of homomorphosis in a heteromorphic anterior regenerate. Induction of heteromorphosis in a homomorphic posterior regenerate. Induction of substrate orientation of nephridia in proximal segments of heteromorphic regenerates. Reorganization of substrate metameres into two or three segments with characteristics of regenerate metameres.

Structure of inhibited buds and cephalic monstrosities indicates brain of *P. millardi* does not have an important role as an organizer in early stages of regenerative growth.

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STUDIES ON THE COMMON BAMBOO-BORER, *DINODERUS OCELLARIS* STEPH.*

Part III. Biology

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1. Introduction

The bamboo-borer, *Dinoderus ocellaris* Steph. is a serious pest of bamboos in India and hitherto no detailed work has been described on the biology of this pest. In Parts I and II of this series of papers[1, 2] a short account of the egg, sex differentiating structures of the pupæ and a technique for rearing larvæ under laboratory conditions have been described. Apart from Beeson's account[3] on the general biology of this pest and a short note on the subject by Sen,[7] no definite information has been available hitherto on the mode of oviposition, the egg-laying capacity of the beetles, the number and the duration of the larval instars, etc. In order to devise ways and means for the effective control of this pest, a systematic study of the biology of this pest was undertaken in these Laboratories and the results are described in the present paper. For the sake of providing complete information, short summaries of work described earlier in Parts I and II[1, 2] have been included at appropriate places.

2. Systematic Position

D. ocellaris Steph. belongs to the order *Coleoptera*, sub-order *Polyphaga*, superfamily *Bostrichoidea* and family *Bostrychidæ*. Lesne[5] included it in the subfamily *Dinoderinæ*, but Böving and Craighead[6] considered *Psoidæ* (including *Dinoderus*), *Bostrychidæ* and *Lyctidæ* as distinct families and made two groups—the first consisting of *Lyctinæ*, *Psoinæ*, *Dinoderinæ* and *Apoleoninæ* and the second *Bostrychinæ*.

3. Type of Damage

The beetles attack bamboo culms within 24 hours of felling by boring in at cut ends. The entrance tunnel is extended towards the centre in a solid culm and between the walls in a hollow culm, where the fibrovascular bundles are widely spaced and there is an abundance of the

* Parts I & II of this series were reported previously under the caption "Studies on the *Dinoderus* Borer in Bamboo".

nutritious pithy tissue. This tissue contains starch and the richer it is in starch the heavier is the attack. The beetles enter longitudinally but bore transversely after a short distance from the entrance holes. Sen[7] has reported that " the entry hole of the adult beetle is carried in a zig zag fashion, more horizontal than vertical and is always found full of frass ".

4. Life-History

(i) *Precopulation period.*—Adults mate outside the bamboos within 18 hours after emergence,[1] one mating being sufficient for the fertilisation of all the eggs laid by a female.

(ii) *Preoviposition period.*—The interval between copulation and oviposition shows much variation depending upon the type of food available. Eggs were observed on the third or fourth day, when freshly emerged adults were released on bamboo-strips (3″ long, 1″ wide and $\frac{1}{3}$″ radius of curvature) having high starch content. In crushed maize, which is not the natural food of the beetles, though it provides a suitable oviposition medium,[4] the preoviposition period varied from 7–16 days.

In bamboo-strips, eggs were observed at the site of the entrance holes when thin layers of the strip were removed. According to Sen,[7] the eggs were found lying burried in the moist and soft frass towards the end of the tunnel. In maize, the eggs were laid loosely in the powder resulting from the beetles boring into the grains. Table I records the observations on the preoviposition period.

Table I. *Preoviposition period of* D. ocellaris *Steph.*

Temp. 86°F. / R.H. 75%

Bamboo-strips			Maize		
Copulating adults released on	Eggs observed on	Preoviposition period (in days)	Copulating adults released on	Eggs observed on	Preoviposition period (in days)
13-9-1947	16-9-1947	3	3-3-1948	16-3-1948	13
16-9-1947	20-9-1947	4	6-3-1948	22-3-1948	16
20-9-1947	24-9-1947	4	11-3-1948	18-3-1948	7
23-9-1947	27-9-1947	4	15-3-1948	29-3-1948	14
26-9-1947	29-9-1947	3	18-3-1948	28-3-1948	10
12-3-1948	16-3-1948	4	20-3-1948	31-3-1948	11
Average ..		3·7			11·8

(iii) *Location of eggs*: *oviposition period.*—Eggs are deposited singly into the ends of the severed fibrovascular bundles (Plate VI). It is probable that this is the usual method of oviposition in bamboos since the beetles did not oviposit either in bamboo dust filled in glass tubes or packed between two glass plates. The distance from the entrance hole and the site of oviposition is about 0·67 mm.

For the determination of the number of eggs laid by the female and the oviposition period, beetles (pairs) were released in small Petri dishes containing crushed maize grains and counts of eggs were made daily. In bamboo strips, a *single female* was released on each and thin layers of the strip were then removed by a sharp razor and the larvæ present (both living and dead) were collected at regular intervals, care being taken not to disturb the female beetle. Since it was not possible to count the exact number of eggs laid by a female beetle, the total number of larvæ collected as above gave an approximate indication of the number of eggs laid. Tables II and III record data on the egg-laying capacity of *D. ocellaris* Stpeh. in bamboo strips and crushed maize grain respectively.

It would appear, therefore, that the number of eggs laid ranges from 50–52 when the food is bamboo. In the case of crushed maize, the number of eggs laid is variable and in any case less than that laid on bamboo strips. The duration of the oviposition period ranges from 12–16 days in the case of crushed maize, and from 46–51 days when the food is bamboo.

TABLE II. *Egg-laying capacity of* D. ocellaris *Steph.*

Food ..... Bamboo Strips

Temperature ..... 86° F. / R.H. 75%

Single female after copulation released on	Oviposition began on	Strips examined on and number of larvæ removed											Total No. of eggs laid by inference
		29·3	30·3	5·4	8·4	12·4	16·4	20·4	24·4	28·4	1·5	6·5	12·5	
12-3-1948 ..	16-3-1948	13 (1)	3	4	5 (1)	3	6	2 (1)	3 (1)	2 (2)	1	3 (1)	Adult dead	52
13-3-1948 ..	*	15	6	2	3 (1)	1	7	6 (1)	2	4	1	2	Adult dead	51
13-3-1948 ..	*	11	8 (1)	5	1	2 (1)	6	4	1	4 (1)	7	Adult dead		52

* Not observed.

Figures in brackets denote number of larvæ found dead.

TABLE III. *Egg laying capacity of* D. ocellaris *Steph.*

Food ..... Crushed maize grains
Temperature ..... 86° F. / R.H. 75%

Copulating adults released on	Oviposition began on	Number of eggs per day																	Total No. of eggs laid
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
3-3-1948..	16-3-1948	3	2	4	1	5	..	7	2	1	..	..	4	..	♀	(dead)			29
6-3-1948..	22-3-1948	2	5	3	6	4	1	5	4	2	1	..	2	3	2	1	♀	(dead)	41
11-3-1948..	18-3-1948	1	3	2	5	1	3	6	3	1	4	2	8	5	..	2	1	♀ (dead)	47
15-3-1948..	29-3-1948	4	2	1	5	7	6	2	1	5	1	3	2	3	2	1	♀	(dead)	45

(iv) *Post-oviposition period.*—Females which failed to oviposit after having laid eggs regularly were considered as having passed the oviposition period. The post-oviposition period varied from 6–7 days at 86° F. and 75% R.H.

(v) *The egg.*—The egg has been described earlier.[1] The egg of *Dinoderus minutus* is 0·81 mm. long and 30·20 mm. broad.[4]

(vi) *Incubation period.*—The incubation period of 46 eggs was studied at 86° F./75% R.H. It varied from 3–5 days and the average was 4·2 days. Sen[7] has reported that the incubation period of 20 eggs ranged between 2 to 4 days at a temperature of 84° F. The incubation period of the eggs of *D. minutus* varies from 3–7 days.[4]

(vii) *Hatching.*—The mode of hatching is rather peculiar. The larva breaks through the broader end of the egg with the tip of its abdomen, which is armed with three dorsally located spines. The chorion is almost completely cut open and the broader end remains fastened like a lid. The larva gradually wriggles out and the hatching is completed within 24 hours.

(viii) *The larva.*—The freshly hatched larva is pale white in colour and almost cylindrical in shape and does not possess a greatly enlarged thorax—a characteristic of the Bostrychid larva. Head is slightly curved and the body is sparsely clothed with hairs. The larva assumes the shape of a grub only after the second moult.

(*a*) *Moulting.*—Forty-eight hours before moulting, the larva becomes completely inactive and ceases to feed. The skin of the body looks dry and

dull. The cuticle ruptures on the median line of the prothorax and the larva wriggles out by muscular contractions of the body. When the moulted skin is adhering to the last few abdominal segments, the larva curves and detaches itself from the skin by holding it in its mandibles. The mandibles of the freshly moulted larva are light brown becoming dark brown in 48 hours.

(*b*) *Larval instars.*—According to Beeson[3] the number of larval instars in *Dinoderus* spp. is at least three and probably four. Investigations carried out on the number of larval instars have now shown that there are eight distinct larval instars.

Freshly hatched larvæ could not be reared in (narrow bore) glass tubes filled with fine bamboo dust or in tunnels bored in the vascular bundles near the site of oviposition or in holes bored in thin bamboo strips, though grown-up larvæ (5th instar) could be easily reared in glass tubes.[2] A few first instar larvæ were, however, reared successfully on the fine powder resulting from the beetles boring into maize grains. This powder (quite distinct from excretory pellets) was found to be rich in starch.

First instar larvæ were kept in small glass tubes (1″ long and $\frac{1}{2}$″ wide), containing a little quantity of this food and the tubes were plugged with cotton and stored in desiccators at 86° F. and 75% R.H. Larvæ were examined daily under a binocular microscope and after every moult they were transferred to separate tubes containing fresh food material. The exuviæ were mounted on slides and the width of the mandibles was measured. Observations based only on those larvæ which moulted more than four times are recorded in Tables IV and V. It will be seen from the measurements of the mandibles that there is a distinct increase in size from instar to instar.

Since only a small percentage of larvæ could be reared successfully by this method, a second method for rearing the larvæ was adopted, and in this the larvæ were provided with their natural food, *viz.*, bamboo strips. Freshly emerged adults (one male and one female in each case) were released on eight bamboo strips (3″ long, 1″ wide and $\frac{1}{3}$″ radius of curvature) kept in separate Petri dishes. The bamboo strips were examined at intervals of 1, 2, 3, 4, 5, 6, 7, 8 weeks from the dates on which the beetles bored inside. Larvæ found each week were removed and their head capsules and mandibles were measured. Observations recorded in Table VI would show that there are eight distinct larval instars and that the increase in the width of the head capsules from instar to instar follows a definite ratio (1 : 1·12).

TABLE IV. *Number and duration of larval instars in* D. ocellaris *Steph.*

Food ..... Crushed Maize

Temperature ..... 86° F. / R.H. 75%

Hatched on	1st moult	No. of days	2nd moult	No. of days	3rd moult	No. of days	4th moult	No. of days	5th moult	No. of days	6th moult	No. of days	7th moult	No. of days	Pupated	No. of days	Adult emerged
20—4—1948..	22-4-48	2	28-4-48	6	5-5-48	7	13-5-48	8	20-5-48	7	27-5-48	7	..	..	..	..	..
8—7—1948..	10-7-48	2	17-7-48	7	25-7-48	8	3-8-48	9	11-8-48	8	..	..	..	..	..	..	..
9—7—1948..	11-7-48	2	18-7-48	7	26-7-48	8	3-8-48	8	11-8-48	8	19-8-48	8	27-8-48	8	..	..	..
29-12-1948..	31-12-48	2	8-1-49	8	16-1-49	8	25-1-49	9	1-2-49	7	9-2-49	8	18-2-49	9	26-2-49	8	1-3-49
31-12-1948..	2-1-49	2	10-1-49	8	17-1-49	7	25-1-49	8	1-2-49	7	8-2-49	7	17-2-49	9	25-2-49	7	28-2-49

TABLE V

Width of mandibles of larvæ of different instars

No. of instars	Measurement of mandibles (mm.)
1 ..	0·066
2 ..	0·093
3 ..	0·106
4 ..	0·133
5 ..	0·159
6 ..	0·186
7 ..	0·199
8 ..	0·213
Pupa ..	0·226
Adult ..	0·239

TABLE VI

Width of mandibles and head capsules of larvæ of D. ocellaris *Steph.*

Food – Bamboo Strips. Temp. 86° F./R.H. 75%

No. of instar	Width of mandibles in (mm.)	Average	Width of head capsules in (mm.)	Average
1	0·064, 0·066, 0·064	0·092	0·51, 0·53, 0·52	0·52
2	0·091, 0·093, 0·093	0·092	0·58, 0·58, 0·58	0·58
3	0·103, 0·105, 0·105	0·104	0·64, 0·65, 0·64	0·64
4	0·133, 0·135, 0·133	0·134	0·73, 0·74, 0·74	0·74
	0·135		0·74	
5	0·156, 0·156, 0·158	0·157	0·83, 0·84, 0·83	0·84
	0·159		0·85	
6	0·184, 0·186, 0·187	0·186	0·93, 0·94, 0·94	0·94
7	0·199, 0·197, 0·199	0·197	1·06, 1·05, 1·06	1·06
	0·196, 0·195, 0·195		1·06, 1·05, 1·06	
8	0·211, 0·213, 0·213	0·212	1·17, 1·19, 1·19	1·18
	0·212		1·18	

The total larval period varies from 56–59 days at 86° F. and 75% R.H., but Sen[7] found that the larval period ranged approximately between 26 and 45 days at 84° F. Each larval instar occupies 7–9 days, excepting the first which lasts only 2 days. Larvæ of *D. minutus*[4] reared in small pieces of bamboo had a feeding period of 41 days and they moulted four times.

(ix) *The pupa.*—Pupation occurs at the end of the larval tunnel and pupæ are found covered with fine frass. The colour of the freshly formed pupa is glistening white, changing to dull yellow with age. The pupa is of about the same size as the last instar larva and is of the exarate type. Mandibles, maxillæ and labium are recognisable and are of the chewing type. Prothorax is large and distinct from mesothorax. Wing pads are elytra-like, with few veins. The pupæ show well-defined and constant sex-differentiating structures,[2]

The pupal period lasts from 3–3·5 days at 86° F. and 75% R.H., the average of 12 distinct observations being 3·2. The pupal period as reported by Sen[7] varied from 4 to 5 days at 84° F.

(x) *The adult.*—The adult spends 3–7 days in the pupal chamber before emerging. The freshly emerged adult is cylindrical in shape, 3–4 mm. long and pale brown in colour, turning dark brown in 72 hours. Head is deflexed and covered by the hood-shaped pronotum, which bears rows of small serrate projections. The antennæ are club-shaped. No secondary sexual characters can be distinguished. Beetles are active fliers and are attracted to light.

(xi) *Number of generations.*—Under laboratory conditions of temperature and relative humidity ranging from 80°–95° F. and 60–85% respectively during the year, three generations were recorded when the beetles were reared on bamboo pieces having high starch content. The first generation starts in March and is completed in 12 weeks, the second runs from June to September and the third, starting in September, overwinters as larvæ and pupates in the last week of February. In case of *Dinoderus minutus*[4] no distinct generations have been discernible throughout the year at Mayaguez, Puerto Rico.

5. Summary

Adult beetles infest harvested bamboo culms within 24 hours of felling by boring in at the cut ends. Eggs, which are elongate, elliptical, 1·23 mm. long and 0·16 mm. broad, are inserted singly into the ends of the severed fibrovascular bundles. A female lays 29–52 eggs in 12–52 days depending on the food material on which it feeds. Incubation period averages 4 days. There are eight larval instars; the first one occupies 2 days and the others take 7–9 days each. The total larval period varies from 56–59 days. Pupal period is 3 days and the pre-emergence period 3–7 days. From egg to the emergence of adult it occupies 60–63 days. There are three distinct generations in a year.

6. Acknowledgement

The author desires to thank Dr. T. S. Subramanian, Superintendent of the Laboratories, for the interest in the work. Grateful acknowledgement is made to the Forest Research Institute, Dehra Dun, for the identification of the insect species as *Dinoderus ocellaris* Steph. This paper is published with the permission of Director of Technical Development and Master General of Ordnance, Army Headquarters, New Delhi.

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An egg of *Dinoderus ocellaris* Steph. is visible in the vessel of a bamboo piece split longitudinally. The adult beetle is also seen in a cell.

ADDITIONS TO FUNGI OF MADRAS—XI

By T. S. Ramakrishnan, F.A.Sc.
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Received September 6, 1950

Plasmopara wildemaniana, P. Henn.

Saccardo, P. A. .. *Syll. Fung.*, **21**, 861, 1912.

On living leaves of *Peristrophe bicalyculata* Nees (Acanthaceæ), Coimbatore, 20–1–49, N. V. Sundaram.

This fungus was observed to be widespread in November in Coimbatore on *Peristrophe bicalyculata*. The imperfect state alone was present. Oospores were not seen.

Helotium malloti Ramakrishnan and Srinivasan, sp. nov.

Apothecia hypophyllous; on veins and along margins of straw-coloured spots, subsessile, powdery, cup-shaped, margin involute, up to 1·0 mm. in diam., dark brown, isolated or in groups; asci cylindric-clavate, 8-spored, hyaline, 150–190 × 17–22 μ, paraphysate, paraphyses filiform, swollen and lobed at the apex, forming a dark brown epithecium; ascospores oblong to elliptical, uniseriate, subhyaline, one-celled, 17–22 × 13–15 μ.

Apothecia hypophylla, subsessilia, exteriora pulverulenta, cupulata, margine involuta, usque 1·0 mm. diam., atrobrunnea, sparsa vel gregaria; asci cylindraceo-clavati, octospori, hyalini, 150–190 × 17–22 μ, paraphysti, paraphysibus filiformibus, ad apicem incrassatis et lobulatis, epithecium fusce brunneum formantibus; ascosporidia oblonga vel elliptica, uniseriata, subhyalina, unicellulata, 17–22 × 13–15 μ.

On living leaves of *Mallotus albus* M. Arg. (Euphorbiaceæ) Shembaganur, 22–12–49. K. V. Srinivasan and T. S. Ramakrishnan.

The spots are irregular and straw-coloured in the initial stages but the central portions drop off soon, leaving holes in the leaves. The apothecia develop on the margins of these holes and along the neighbouring veins either singly or in small groups of 3–4. A brown stromatic mass is embedded in the tissues of the leaf sometimes extending throughout the thickness. The stroma bursts through the lower surface and the apothecium is borne on this. It is almost sessile, deep saucer-shaped or sometimes obconical with incurved margin and presents a brown powdery surface. The asci are closely

packed interspersed with paraphyses which are longer than the asci. The apices of the paraphyses are enlarged and provided with numerous digitate lobes. These are closely packed forming a brown epithecium. On moistening, the epithecium becomes gelatinous and sticky. The interlocking apices of paraphyses do not easily separate.

Pseudophacidium photiniæ Ramakrishnan and Srinivasan, sp. nov.

Spots amphigenous, stroma-like, dark violet, circular or irregular; apothecia hypophyllous in the middle of the spot, erumpent, discoid, sessile, black, umbonate, upto 1·5 mm. across; asci clavate or cylindric, hyaline, 8-spored, 90–150×9–14 μ, paraphysate, paraphyses filiform, sometimes branched, swollen at the tip; ascospores hyaline, oblong, uniseriate or irregularly biseriate, one celled, 10–14×5–7 μ.

Maculæ amphigenæ, stromatoideæ, atroviolaceæ, orbiculares vel irregulares; apothecia hypophylla, in maculæ centro disposita, erumpentia, discoidea, sessilia, atra, umbonata, usque 1·5 mm. lat.; asci clavati vel cylindracei, hyalini, octospori, 90–150×9–14 μ, paraphysati, paraphysibus filiformibus interdum ramosis, ad apicem incrassatis; ascosporidia hyalina, oblonga, monosticha vel irregulariter disticha, unicellata, 10–14×5–7 μ.

On living leaves of *Photinia notoniana* W. and A. (Rosaceæ). Kodaikanal, 22-12-49, T. S. Ramakrishnan and K. V. Srinivasan.

Round or irregular dark violet isolated spots are formed on both sides of the leaf. The spots are raised due to the development of a subepidermal stroma. In the centre of each spot, hypophyllously is a black erumpent discoid apothecium with an umbonate projection in some. Apothecium is formed on the veins also. It is sessile with a fairly thick hypothecium, opening out after bursting through the epidermis into an effuse structure. Sometimes an air cavity is formed in the hypothecium. A definite epithecium is absent. The asci are clavate and rounded at the apex. Numerous paraphyses are present; these are sometimes branched at the base. The apices are slightly swollen and enveloped in mucilage. Very old apothecia may bear subhyaline spores.

Eudarluca indica, sp. nov.

Perithecia in clusters, erumpent, black, 120–180 μ in diam., ostiolate, immersed in a stroma of sooty coloured closely packed pseudoparenchymatic context; asci cylindrical, hyaline, 8-spored, 65–75×6–7 μ, paraphysate; ascospore fusiform, uniseriate, hyaline, with one prominent septum, 16–22×4–5 μ.

Perethecia gregaria, erumpentia, nigra, 120–180 μ diam., ostiolata, immersa in stromatæ texturæ densæ, pseudoparenchymaticæ; asci cylindrici, hyalini, octospori, 65–75 $\times$ 6–7 μ, paraphysati; ascosporidia fusiformia, uniseriata, hyalina, prominenter uniseptata, 16–22 $\times$ 4–5 μ.

Infecting uredosori of *Uredo amomi* Petch or *Amomum*, sp., Anamalais, 22-11-50, T. S. Ramakrishnan.

The perithecia are amphigenous, black and occur in groups of five to ten, infecting the uredia. A thick stroma of small pseudoparenchymatous cells occupies the thickness of the leaf. The perithecia are sunk in this stroma and protrude slightly above the surface as black globose bodies. The ostiole is recognisable in some of the perithecia. The asci are basal and are mixed with filiform paraphyses. The ascospores are broad in the middle and tapering towards the ends. Only one septum is clearly visible in the centre. *E. australis* Speg. has been recorded on *Uredo cannæ* Wint. from Brazil. Hansford (1946) describes *E. australis* as occurring singly or close clusters in the sori of many *Uredineæ* and being aparaphysate. Doidge (1941) states that the basal stroma grows into the mesophyll of the leaf and that the paraphysoids are sparse. In the specimens under study the stroma occupies the thickness of the leaf and paraphyses are seen. The fungus comes close to *E. australis*.

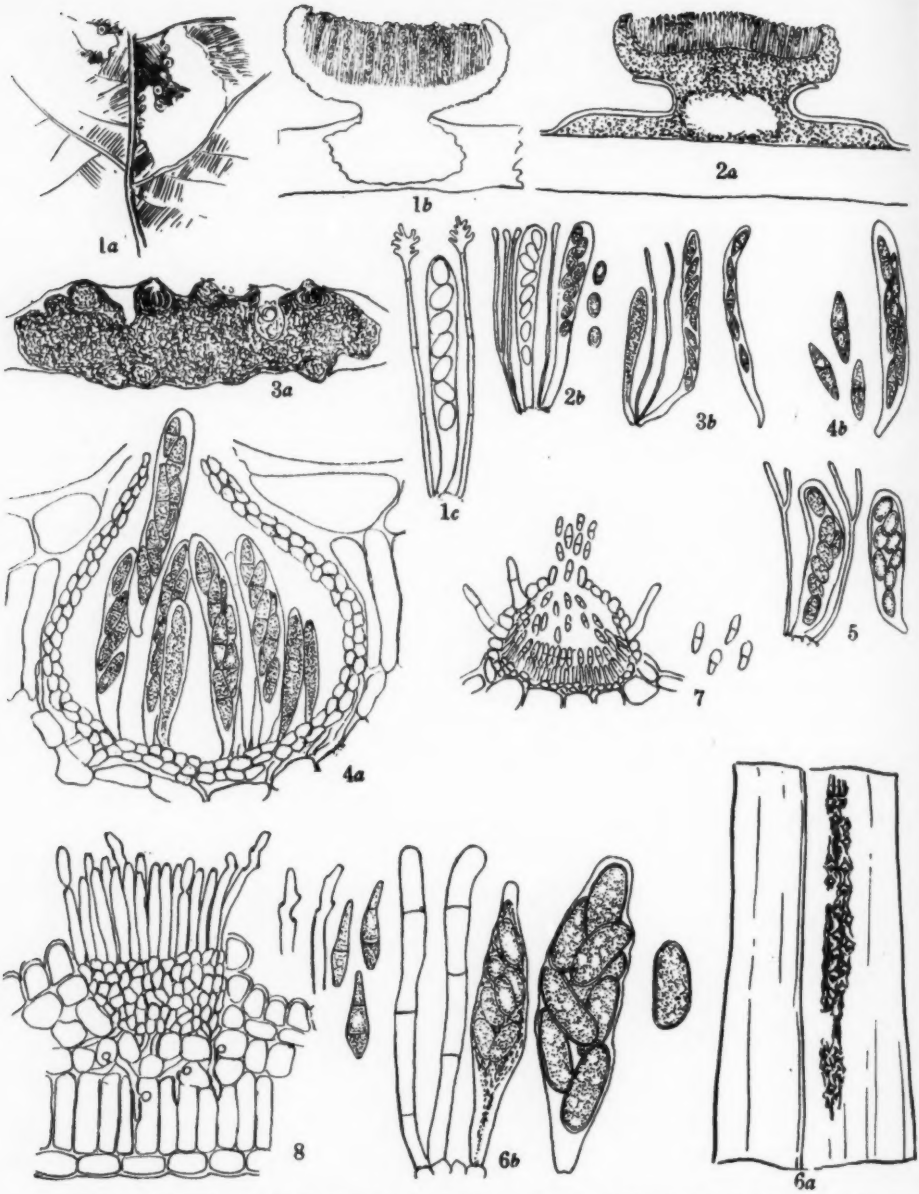
Mycosphærella melothriæ sp. nov.

Spots amphigenous, prominent hypophyllously, circular to irregular, dull green; perithecia minute, crowded, subepidermal, ostiolate, black, aparaphysate; asci cylindrical to clavate, hyaline, 8-spored, 52 $\times$ 13 μ (36–72 $\times$ 9–15); ascospores irregularly biseriate, 2-celled, fusiform, hyaline, 19 $\times$ 6 μ (15–24 $\times$ 5–7).

Maculæ amphigenæ, prominenter hypophyllæ, orbiculares vel irregulares, dilute virides; perithecia minuta, gregaria, subepidermalia, ostiolata, atra, aparaphysata; asci cylindrici vel clavatis hyalini, octospori, 52 $\times$ 13 μ (36–72 $\times$ 9–15); ascosporidia irregulariter biseriata, uniseptata, fusiformia, hyalina, 19 $\times$ 6 μ (15–24 $\times$ 5–7).

On living leaves of *Melothria mucronata* Cogn. (Cucurbitaceæ), Ootacamund, 10–10–49, T. S. Ramakrishnan.

Indefinite roundish dull green spots are formed on the leaves. These are more prominent on the lower surface and are studded with numerous minute black perithecia. Associated with the perithecia are pycnidia of a *Septoria*. These are subepidermal with elongated, cylindrical, hyaline, pycnidiospores measuring 70 $\times$ 3 μ (51–105 $\times$ 3).



FIGS. 1–8. Fig. 1 (*a*) Portion of a leaf with apothecia of *Helotium malloti*; (*b*) Section through an apothecium (semidiagrammatic); (*c*) ascus and paraphyses, × 250. Fig. 2 (*a*)

Section of the apothecium of *Pseudophacidium photiniæ* (diagrammatic); (*b*) asci, poraphyses and ascospores, × 250. Fig. 3 (*a*) Section of stroma of *Eudarluca indica* (diagrammatic); (*b*) asci and paraphyses. Fig. 4 (*a*) Section of perithecium of *Mycosphærella melothriæ*; (*b*) ascus and ascospores, × 375. Fig. 5. Asci and paraphyses of *Physalospora achyranthis*, × 250. Fig. 6 (*a*) Bit of a leaf showing stromata of *Sphærodothis coimbatorica*; (*b*) asci, paraphyses and a spore, × 250. Fig. 7. Section of a pycnidium of *Ascochyta infortunata* and spores, × 250. Fig. 8. Section of leaf showing *Cercospora pulchella*, × 250.

Guignardia nilagirica Ramak., T. S. and K.

Ramakrishnan, T. S. and K. .. *Proc. Ind. Acad. Sci.*, **28**B, 56, 1948.

On living leaves of *Derris scandens* Benth. (Papilionatæ), Walayar, Malabar, 15–9–49, K. Ramakrishnan.

Indefinite light green spots are formed on the leaflets. These are uniformly studded with the red spermagonia and black perithecia of the fungus. This is a new host for this fungus.

Physalospora achyranthis sp. nov.

Spots indefinite; perithecia amphigenous, innate erumpent, black, up to 200 μ broad, isolated, ostiolate; asci hyaline, clavate, with a small foot, 80–95 × 21–26 μ, paraphysate, paraphyses septate, branched; ascospores 8, irregularly biseriate, oblong to obovate, hyaline, one celled, 17–22 × 6–10 μ.

Maculæ indefinitæ; perithecia amphigena, innata, erumpentia, atra, usque 200 μ lat., isolata, ostiolata; asci hyalini, clavati, brevi pediculo insidentes, 80–95 × 21–26 μ, paraphysati, paraphysibus septatis, ramosis; ascosporidia 8, irregulariter biseriata, oblonga vel obovata, hyalina, unicellata, 17–22 × 6–10 μ.

On living leaves of *Achyranthes aspera* L. (Amarantaceæ), Kodaikanal, 26–12–49, T. S. Ramakrishnan.

Indefinite yellowish green areas mark the affected regions of the leaves. Studded in these patches and visible on both sides are numerous black round dot-like perithecia. The asci are short and stout with the apical portion thicker than the sides. Paraphyses are limited in groups and branched in the upper portions. The ascospores are arranged in two irregular rows. Associated with the perithecia are spermagonia producing minute hyaline, bacillar spores.

Sphærodothis coimbatorica, sp. nov.

Stromata foliicolous, epiphyllous, upto 40 mm. long and 5 mm. broad, black, with many loculi, subepidermal; perithecia ostiolate; asci many clavate, thin-walled, hyaline, 100–145 × 27–40 μ, paraphysate, paraphyses

septate; ascospores 8, distichous, oblong or reniform, rounded at ends, 32–43 × 13–20 μ, golden brown in colour, one-celled.

Stromata foliicola, epiphylla, usque 40 mm. longa et 5 mm. lata, atra, subepidermalia, loculis numerosis; perithecia ostiolata; asci plures, clavati, membrana tenui ornati, hyalini 100–145 × 27–40 μ, paraphysati, paraphysibus septatis; ascosporidia 8, biseriata, oblonga vel reniformia, apice rotundata, 32–43 × 13–20 μ, aureobrunnea colore, unicellata.

On living leaves of *Calamus rotang* L. (Palmæ), Anamalais, 22–2–50, T. S. Ramakrishnan.

The stromata form elongated raised black formations on the leaflets. Bursting through the stroma and arranged in one or two lines are several black mammiform perithecia. Each perithecium is deeply sunk in the stroma which is composed of vertically arranged cells. The paraphyses and the ascus wall become gelatinised as the perithecia mature. In old loculi groups of ascospores are found to be surrounded by a gelatinous mass without any ascus wall. Young spores are hyaline but they become golden brown when old. Theissen and Sydow (1915) and H. and P. Sydow (1917) have recorded *Phæochora calamigena* (B. et Br) Theiss. and Syd. on *C. rudentum* from Ceylon and *C.* sp. from the Philippines respectively. The measurements of the asci and ascospores in the two are almost equal. But the fungus under study is not *Phæochora* as well developed paraphyses are present. Therefore it is different and is included under *Sphærodothis* which differs from *Phæochora* in the possession of paraphyses.

Entyloma irregulare Johans.

Clinton, G. P. .. *North American Ustilaginaceæ, Pro. Bost. Soc. Nat. Hist.* No. 9, 458, 1904.

On living leaves of *Poa annua* L. (Gramineæ), Ootacamund, 3–10–49, N. V. Sundaram.

Two leaf smuts *E. crastophilum* Sacc. and *E. irregulare* have been recorded on *Poa*. In the smut under study the sori are circular and the spores are often irregular being occasionally elongated. Though the two species are considered to be probably synonymous by some, Clinton is in favour of keeping them apart. Conidia were not observed in the specimen collected.

Uromyces sojæ (P. Henn.) Syd.

Sydow, H. and P. .. *Monographia uredeinearum*, **2**, 128, 1910.

On living leaves of *Glycine max* (Linn.) Merr. (Soyabean) (Papilionatæ) grown at the Agricultural Research Station, Palur (South Arcot), 15–1–50, M. A. Sankara Iyer.

Butler and Bisby (1931) state that no rust on *Glycine soja* has in reality been found in India. There is no doubt about the host plant now collected.

Ascochyta infortunata sp. nov.

Spots indefinite; pycnidia minute, amphigenous, globose, black, erumpent, ostiolate, $97 \times 88\,\mu$ (78–120 × 59–99); spores hyaline or subhyaline, linear oblong, one septate, $9 \times 5\,\mu$ (5–13 × 5–6).

Maculæ indeterminatæ; pycnidia minuta, amphigena, globosa, atra, erumpentia, ostiolata, $97 \times 88\,\mu$ (78–120 × 59–99); sporæ hyalinæ vel subhyalinæ, lineari-oblongæ, 1-septatæ, $9 \times 5\,\mu$ (5–13 × 5–6).

On living leaves of *Clerodendron infortunatum* L. (Verbenaceæ) Kallar (Coimbatore), 25–6–50, T. S. Ramakrishnan.

The diseased leaf exhibits slight distortion with revolute margin. Definite spots are wanting but the infected portions are paler in colour. Pycnidia are present on both sides of the leaves but more numerous on the under side. They are innate in origin but burst through and appear as black dots on the surface. In some of the pycnidia the ostiole is surrounded by a layer of vertically elongated cells. At the base, varying numbers (3–5) of setose structures are formed on the surface in some of the pycnidia. These are septate and unbranched. The spores are produced on short unbranched stalks. They are mainly linear oblong but in some become variously swollen to give different shapes. The septum is clear but constriction at the septum is not common. Normally the spores are hyaline but sometimes a light tinge of olive is evident. The wall of the pycnidium is membraneous.

Cercospora ferruginea Fuckel.

Saccardo, P. A. .. *Syl. Fung.*, **4**, 444.

On living leaves of *Artemesia vulgaris* L. (Compositæ), Anamalais, 15–2–50, T. S. Ramakrishnan.

Infection was widespread on the plants which were being grown in hedges. The characteristic yellowish green spots appear on the upper surface. Corresponding to these, soot-coloured growths of fungus are present on the lower surface.

Cercospora pulchella sp. nov.

Spots amphigenous, minute, angular, brown; conidiophores hypophyllous, unbranched, non-septate, in dense clusters, light brown, apex geniculate, $96 \times 8\,\mu$ (50–167 × 5–11); conidia obclavate, light brown, 2–3 celled, $49 \times 11\,\mu$ (31–78 × 8–13).

Maculæ amphigenæ, minutæ, angulatæ, brunneæ; conidiophori nec ramosi, nec septati, dense-fasciculati, leviter-brunnei, apice geniculati, 96×8 μ (50–167×5–11); conidia obclavata, leviter brunnea, 2–3 cellata, 49–11 μ (31–78×8–13).

On living leaves of *Indigofera pulchella* Roxb. (Papilionatæ) Kodaikanal, 24-12-49, K. V. Srinivasan.

Many spots develop on the leaflets followed by defoliation. The conidiophore tufts are on the lower surface. The stromata are substomatal in origin but the epidermal cells are pushed apart very soon and the conidiophores project out. Septa are not present in the conidiophores. The tips are geniculate and three or more scars of spore attachment are seen on each.

Tuberculina persicina (Ditm.) Sacc.

Saccardo, P. A. .. *Syll. Fung.*, **4**, 653, 1886.

Infecting the æcia of *Aecidium pavettæ* Berk. and *Aecidium* sp. on *Strobilanthes cuspidatus* T. And., Burliar (Nilgiris), 15–10–49, T. S. Ramakrishnan and K. V. Srinivasan.

Violet coloured rounded powdery growths developed in the æcia completely obliterating the rust. The sporodochia were made up of closely arranged conidiophores measuring 44×5 μ (30–51×4–6). The conidia were unicellular, light tinted, globose and measured 8·0 μ in diameter. Almost all the æcia were infected.

I am indebted to Dr. H. Santapau of St. Xavier's College, Bombay, for the Latin translations.

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FLOWERING HABITS OF *DICHANTHIUM ANNULATUM*, Stapf., *DICHANTHIUM CARICOSUM*, A. Camus AND *BOTHRIOCHLOA INTERMEDIA* (Br.) A. Camus

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A KNOWLEDGE of the flowering habits of grasses is of prime importance to a grass breeder in devising suitable methods of controlled pollination. Observations on the flowering habits of *D. annulatum*, *D. caricosum* and *B. intermedia* are described in this note, as no such work appears to have been done previously on these species.

The time interval between the various stages commencing with the appearance of the flag-leaf of the boot to complete anthesis of the panicles in the above three species was studied. This information was required for purposes of selfing and bulk emasculation which involves bagging in the former case and hot-water treatment in the latter prior to anthesis. Bagging done earlier resulted in the boot tearing through and growing out of the bags. Hot-water treatment given early in the boot stage gave no useful results.

REVIEW OF LITERATURE

Grasses bloom, *i.e.*, extrude their stamens and liberate pollen most abundantly in the early morning. This is an almost universal rule (Beddows, 1931) although the period of flowering may be delayed and prolonged by cloudy atmospheric conditions. For several grasses there is apparently a second less intensive anthesis period in the afternoon. Fruwirth (1916) found a secondary blooming period in the afternoon that lasted for one or two hours. Sando (1936) found in the *Agropyron elongatum*, (Host) Beauv., the maximum anthesis between 6 and 8 A.M. but there was a second wave between 3 and 4 P.M. He observed that blooming was most active when the sun was shining and the temperature was 70° F. or above. No blooming was ever observed at temperatures below 52° F. He believes the delay or reduction in the anthesis caused by a cloudy sky is due more to the lowering of the temperature than to increased humidity. The views of Sando (1936) respecting the effect of humidity are confirmed by the studies of Stephens and Quinby (1934) in *Sorghum*. They concluded: "Relative humidity apparently did not influence the time of blooming".

They found that under field conditions the rate of blooming was highest shortly after mid-night. Further, a relatively small proportion of flowers opened before 10 P.M. or after 8 A.M., but there was no hour in which flowers were never found opening. They also observed that light conditions were a most important factor governing the time of blooming and that lowering the temperature reduced the rate of blooming. Fruwirth (1916) agrees with Stephens and Quinby (1934) regarding the effect of humidity but not as to light. He found that Rye grass and Orchard grass (*Dactylis glomerata*, L.) bloomed in spite of the lack of light. Recent investigations have emphasised the importance of the relative proportion of daylight and darkness in the reproductive processes of plant development. In Orchard grass (*Dactylis glomerata*, L.), Wolfe (1925) found that 76·4% of the flowers bloomed from sunrise to noon, 6·6% from noon to sunset and only 0·3% from sunset to mid-night. Jenkin (1924) found that Tall oat grass [*Arrhenatherum elatius*, (L.) Mert and Koch.] and Timothy (*Phleum pratense*, L.) unlike the perennial rye grass (*Lolium perenne*, L.) and red fescue (*Festuca rubra*, L.) begins blooming early in the morning. Kornicke (1885) reports that in *Sorghum*, flowering begins in the morning between 8 A.M. and 9 A.M. and may continue till evening. Graham (1916) found majority of the flowers of *Sorghum* opening between 2 A.M. and 4 A.M. Ramnathan (1924) found flowering period to be between 1 A.M. and 8 A.M. in *Sorghum*. Kornicke and Ramnathan further found that in *Sorghums* the dehiscence of anthers took place after they became pendant. Graham (1916) observed that it could take place at earlier stages also, in addition. At Coimbatore, Rangaswami Ayyangar and Panduranga Rao (1931) found that the dehiscence of anthers in *Sorghum* was simultaneous with the opening of glumes. They further found that the commencement of anthesis was not uniform but varied from 12 mid-night to 2 A.M. generally and the period between anthesis and dehiscence was 45 minutes.

Material and Methods

The beginning of the reproductive phase in grasses is the impending emergence of the "boot" which is the terminal leaf-sheath. The boot presents a bulged appearance due to its enclosing a panicle or ear-head. The leaf-blade of the boot is known as the "flag-leaf". Prior to emergence of such boots the tops of the stalks of plants assume a characteristic swollen appearance. The boots remain in a swollen condition till the panicles emerge out of them completely.

For the study of the time interval between the various stages commencing with the appearance of the flag-leaf of the boot to complete anthesis of panicles, five plants each of *D. annulatum* (Bushy type), *D. caricosum* and

B. intermedia were selected. Five boots, the flag leaves of which had just made their appearance were selected from each plant for observation. In order to obtain detailed information regarding the blooming process of *D. annulatum*, ten panicles from each of the five plants were selected and the heads marked. Each panicle possessed from 4 to 6 spikes and, therefore, each spike was labelled by means of small round bits of paper obtained from the punching machine. This facilitated the recording of each individual spike. Because of extreme closeness of florets the portions of the spikes over which florets opened were measured by a centimetre scale. Heads were observed at one hour interval both during day and night. Observations at night were made with the aid of a torch. The flower was considered as having bloomed as soon as the palea and the lemma were sufficiently separated so that the stigma and anther were visible. All observations were made on the bushy type of *D. annulatum*. In this type pedicelled florets are neuter. In the other type of *D. annulatum* pedicelled florets are male and the habit of growth is spreading. Field observations were confirmed in the laboratory as follows: Five panicles each of *D. annulatum* and *D. caricosum* with about a foot of stalk were cut and placed in water in a conical flask in the evening about five hours before the commencement of anthesis.

Observations

Data regarding the time interval between the various stages commencing with the appearance of the flag-leaf of the boot to complete anthesis of panicles of *D. annulatum*, *D. caricosum* and *B. intermedia* are presented in Table I below.

Table I. *Periods (in days) for various stages of panicle development beginning with the appearance of flag-leaf*

Stage	*D. annulatum* days	*D. caricosum* days	*B. intermedia*
1 Appearance of flag leaf to complete emergence of boot	6	6	Panicles start coming out of the boots when boots have emerged half way out of the leaf below the flag leaf. This stage is about five days from the appearance of flag leaf. Panicles begin to dehisce bit by bit as they emerge out of the boots thus completing the processes of anthesis and emergence of panicles simultaneously in about four days. Blooming and dehiscence take place during night and early morning as in *D. annulatum*.
2 Emergence of panicle from boot	1	1	
3 From complete emergence of panicle to commencement of anthesis	2	2	
4 Completion of anthesis and dehiscence	2	2	

From the data in Table I it will be seen that in *D. annulatum* and *D. caricosum* a period of two days elapses between complete emergence of panicle out of the boot and the commencement of anthesis. Selfing and bulk emasculation in these species, therefore, can conveniently be done during these two days. Whereas in *B. intermedia* selfing and bulk emasculation have to be done during boot stage about three days after the appearance of flag-leaf and not later as emergence of panicle and anthesis commence simultaneously.

Details of Blooming of the Florets and Dehiscence of Anthers in *D. annulatum*

At the time of complete emergence, the colour of the panicle is pale green. It remains in that condition for one day. Next day the colour changes slowly to purple-red. During the next night, as a rule, complete anthesis takes place. If some florets or portions of spikes remain unopened they open to a very slight extent the following day but the anthesis is completed during the second night.

The florets bloom by opening the glumes slowly at first and then more rapidly. The stigmatic ends start protruding and about the same time the filaments of the stamens begin to elongate. These as a rule elongate beyond the stigmatic ends. About 15 to 20 minutes later the anthers dehisce apically and slowly with the inversion of the anthers the releasing of the pollen takes place. When the anther-sacs are completely emptied they collapse and appear translucent. The time required from the opening of florets to the extrusion and drooping is about an hour.

The data collected showed that anthesis and dehiscence of anthers take place during the night and about 80% of the florets open and dehisce between 9 P.M. and 4 A.M., 4·5% florets open between 5 and 6 A.M. and from 6 A.M. onwards till 8 P.M. only 1% florets open. The remaining ones open during the following night. In every spike blooming and dehiscence generally start from 4th to 6th floret and proceed downwards. This continues till some florets at the bottom remain unopened. Then the bottom and top ends open simultaneously. Florets on some of the panicles which start blooming late in the morning between 4 and 6 A.M. rarely flower during the following day but complete their anthesis during the following night. In some spikes irregular blooming was observed. In these, florets opened at various places on the same spikes at one and the same time.

In the spreading type of *D. annulatum* the pedicelled florets are male and which open during the night, 24 hours after the completion of anthesis

and pollination of sessile florets. The period of blooming and dehiscence of anthers in this type is the same as in the bushy type of *D. annulatum*.

In *D. annulatum*, therefore, anthesis and dehiscence take place as a rule, during night and early morning between 9 P.M. and 4 A.M.

In *B. intermedia* blooming and dehiscence take place during night and early morning as in *D. annulatum* but takes about 4 days to complete emergence of panicle and anthesis of florets which processes go on simultaneously in that species.

Blooming and dehiscence of anthers of *D. annulatum* and *D. caricosum* observed in the laboratory experiment showed the same sequence of processes as those observed in the field. It was observed that *D. caricosum* also blooms and dehisces during night and early morning at about the same time as *D. annulatum*.

Discussion

Since a period of two days elapses between complete emergence of panicle out of the boot and the commencement of anthesis, bagging can conveniently be done and large number of panicles can be bagged without fear of boots tearing through and growing out of the bags. During these two days bulk emasculation can also conveniently be undertaken. In *B. intermedia* selfing and bulk emasculation have to be done during boot stage about three days after the appearance of flag leaf so as to avoid any loss of seed on account of the boots tearing through and growing out of the selfing bags. For all this work butter paper bags, the seams of which had been sealed with paraffin wax were used throughout and were fastened on to the peduncles of panicles after putting a wad of cotton to allow access of air into the bags and at the same time to protect the peduncles from injury likely to be caused by the knots of strings used for tying the selfing bags.

From the observations it appears that in *D. annulatum* pollination between florets of the same panicle is the rule the pollen from the higher and earlier opening florets falling on and pollinating the stigmas of lower florets. Cross-pollination is likely to take place in the florets which open at the commencement of the anthesis. Observations showed that *B. intermedia* also behaves as a self-pollinated species. Laboratory experiment showed that *D. caricosum* blooms and dehisces at about the same time as *D. annulatum* and behaves as a self-pollinated species. Satisfactory seed setting under bags (Oke, *Unpublished*) also lend support to the fact that *D. annulatum* and *B. intermedia* are self-pollinated species.

Observations of Graham (1916) and Rangaswami Ayyangar and Panduranga Rao (1931) in *Sorghum* compare closely with those of *D. annulatum* in that the florets of *Sorghum* open and dehisce during night and a part of the early morning as in the case of *D. annulatum*.

Summary

The time interval between the various stages commencing with the appearance of flag-leaf of the boot to complete anthesis of panicle in *D. annulatum*, *D. caricosum* and *B. intermedia* was studied. Detailed study of blooming of florets and dehiscence of anthers of *D. annulatum* was also carried out.

In *D. annulatum* and *D. caricosum* a period of two days elapses between complete emergence of panicles out of the boots and the commencement of anthesis thus allowing sufficient time to self and treat number of panicles at a time. In *B. intermedia* anthesis and dehiscence take place as the panicles emerge out of the boots and thus it is necessary to bag or treat the panicles immediately three or four days after the appearance of flag-leaf.

A period of 11 days in *D. annulatum* and *D. caricosum* and 9 days in *B. intermedia* is required from the appearance of flag leaf to completion of anthesis.

In *D. annulatum* anthesis and dehiscence take place as a rule, during night and early morning between 9 P.M. and 4 A.M.

In *B. intermedia* anthesis of florets and emergence of the panicles out of the boots take place simultaneously bit by bit and takes about 4 days for completion of these two processes. Blooming and dehiscence, however, take place during night and early morning as in *D. annulatum*.

D. annulatum, *D. caricosum* and *B. intermedia* appear to behave as self-pollinated grass species.

Laboratory experiments showed that *D. caricosum* blooms and dehisces during night and early morning at about the same time as *D. annulatum*.

The author is greatly indebted to Professor L. S. S. Kumar, M.Sc. (Lond.), F.A.Sc., Economic Botanist and Principal, College of Agriculture, Poona, for his guidance and encouragement during the course of this work.

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POLLEN AND EMBRYO-SAC OF TWO EUPHORBIACEÆ

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The family Euphorbiaceæ has received considerable attention from embryologists and as many as eight different types of embryo-sac development has been recorded in this family. The earlier literature has been reviewed by Schnarf[13] and later work by Kajale and Rao,[7] Maheshwari[10] and by Banerji and Dutt,[2] and so it is not reviewed here. Of the important contributions on the subject made since then, mention may be made of Mc Cann's[11] work on the embryology of the Tung oil tree. Swamy and Balakrisinna's[14] investigations on the development of the female gametophyte in *Acalypha tricolor* and Margaret Landes's[8] studies on the development of the seed in *Acalypha rhomboidea*. Maheshwari[9] and later Johansen[6] have recently reviewed the literature on plant embryology in general.

Material and Methods

Pedilanthes tithymaloides Poir and *Gelonium multiflorum* A. Juss., furnished the material on which the present investigation is based. A few observations have also been made on *Phyllanthus reticulata* Poir. Of these plants, *Gelonium multiflorum* is dicœcious, while the other two are moncœcious. All the plants are common in West Bengal.

Flower buds were fixed in Nawaschin's fluid, washed, dehydrated, cleared and embedded in the usual way. Sections were cut at a thickness of 8 to 16 μ and stained either in Heidenhain's iron-alum hæmatoxylin or in Newton's Gentian-violet-iodine stain.

Observations

I. *The Flower.*—In *Pedilanthes tithymaloides* the flowers are encased within a conspicuous red coloured slipper-shaped perianth-like involucre composed of two bracts. On these, the anterior is boat-shaped, larger and almost covers the inflorescence by its incurved margins (Fig. 6). It bears two hemispherical glands at its base. The posterior bract is convex in form and partially covers the anterior at its dorsal surface, leaving an opening at its apex as illustrated in Fig. 6. It develops a pouch-like projection at its base, inside which two hemispherical glands are located (Fig. 4).

The mature glands show a single layer of closely arranged columnar cells on the exterior, followed by another layer of similar cells surrounding a central core of parenchymatous tissue. The gland cells are rich in cytoplasm and each have a centrally situated prominent nucleus (Fig. 5). The trace supplying the gland branches inside. The ultimate branches spread out and consist mainly of spiral vessels.

The male flowers consist of a single stamen which is articulated at a short distance below the anther. A circular depressed area delimits this region. Longitudinal section reveals that the cells of this region are radially elongated, compressed and much smaller in size than the adjoining cells on both sides. The pedical of the female flower, however, shows no such articulation. The female flower consists of a trilocular ovary, composed of three united carpels, a solid style and trifid stigma. A single pendulous ovule occurs in each chamber.

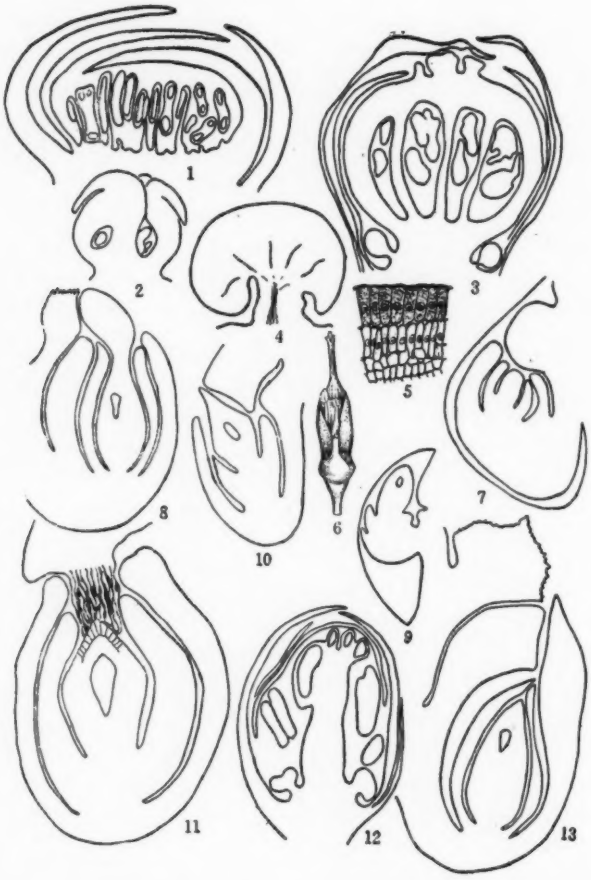
In the development of the inflorescence, the bracts are the first to differentiate, followed by the progressive elongation of the central axis which gives rise to the pistil. The primordia of the stamens (male flowers) are comparatively late in development and arise in acropetal succession. The glands differentiate almost simultaneously with the stamens.

In *Gelonium multiflorum* the male flowers have five sepals which are the first to differentiate and enclose a convex receptacle on which are produced numerous stamens in acropetal succession and more or less simultaneously (Fig. 1). The female flower consists of an equal number of sepals and a tricarpellary ovary with three sessile, capitate and recurved stigmas (Fig. 2). Each chamber of the ovary contains a single pendulous ovule.

Development stages of the flowers of *Phyllanthus reticulatus* have not been studied. Longitudinal sections of the male and female flowers are represented in Figs. 12 and 3 respectively. The presence of conspicuous glands at the base of the gynœcium and the andrœcium appears to be a characteristic feature.

II. *Development of the Microspores.*—The archesporium in *Pedilanthes tithymaloides* is hypodermal in origin. The cells of this layer soon divide to give rise to a parietal layer and the primary sporogenous layer. The parietal layer divides twice in rapid succession, so that four layers of cells are produced below the epidermis. The one next to it forms the endothecial layer and the innermost one next to the sporogenous cells the tapetum. The parietal layers degenerate at an early stage of the development of the microspores.

In *Gelonium multiflorum* the development of the sporogenous cells is similar. The sub-epidermal and the innermost layer, as usual, form the endothecium and the tapetum respectively.



TEXT-FIGS. 1–13.—Fig. 1. L. S. of a staminate flower of *G. multiflorum*, × 15. Fig. 2. L. S. of a pistillate flower of same, × 10. Fig. 3. L. S. of a pistillate flower of *P. reticulatus*, × 15. Fig. 4. Outline of a gland of *P. tithymaloides*, × 15. Fig. 5. L. S. of part of the same × 75. Fig. 6. Inflorescence of *P. tithymaloides* covered up bracts × 2. Fig. 7. Early stage of the development of ovule and obturator in *G. multiflorum* × 75. Fig. 8. L. S. of ovule of *P. reticulatus*; note obturator and nucellar beak, × 30. Figs. 9, 10 & 11. Stages in the development of the ovule and obturator in *P. tithymaloides* × 75. Fig. 12. L. S. of staminate flower of *P. reticulatus*, × 15. Fig. 13. L. S. of mature ovule of *G. multiflorum*; note obturator, ×75.

In both the aforesaid plants the tapetal cells become bi-nucleate during synizesis. Quadrinucleate cells have also been observed and in rare instances trinucleate cells in *Pedilanthes tithymaloides* (Fig. 24). The tapetal cells of *Phyllanthus reticulatus* are bi-nucleate only.

The meiotic divisions present no irregularity. At diakinesis the chromosomes appear as globular bodies in *P. tithymaloides* and rod-like in *G. multiflorum*. During metaphase of division I, eighteen bivalent chromosomes have been observed in the former and eleven in the latter (Figs. 16 and 17). An interkinetic stage intervenes before the II division, which is normal. The pollen quartets are arranged mostly in a tetrahedral manner and cytokinesis takes place by the process of furrowing.

The pollen-grains are binucleate in all the three plants. The generative nucleus appears to be slightly smaller in size and in some instances is noted to be lying at the periphery, being delimited by a cytoplasmic membrane (Fig. 26).

The mature (dry) pollen-grains of *P. tithymaloides* are triangular in form, each side of which measures 25 μ, tricolpate and with three germ pores situated centrally inside the furrows. The exine surface is coarsely granular (Fig. 27). The pollen grains of *G. multiflorum* are spherical in shape, 40 μ in diameter. The exine is closely set with cone-like bodies with rounded ends. The pointed ends being in contact with the exine (Fig. 29).

III. *The Ovule, Integuments, Nucellus and Obturator.*—In *P. tithymaloides* and *G. multiflorum* the ovules arise as minute papillate processes from the upper angle of the placental axis and very soon curve upwards to take up an anatropous form, even before the differentiation of the megaspore mother cells. At this stage a considerable amount of empty space is present at the base of the ovule in the former plant (Fig. 9) but not in the latter. The inner integument is the first to arise followed soon by the outer.

In *P. tithymaloides* the growth of the outer integument is very rapid and covers the nucellus at the megaspore mother-cell stage. It is composed of four layers of cells except at the micropylar region where it is thicker. The inner integument is comparatively massive and is composed of eight to ten layers of cells. In the mature ovule the inner integument lies nearly at the same level as the nucellar beak while the outer integument extends over it (Fig. 11). Both the integuments take part in the formation of the micropyle which is somewhat wide.

In *G. multiflorum* the inner integument is composed of four to eight layers of cells, the thickness being variable at different regions. It becomes

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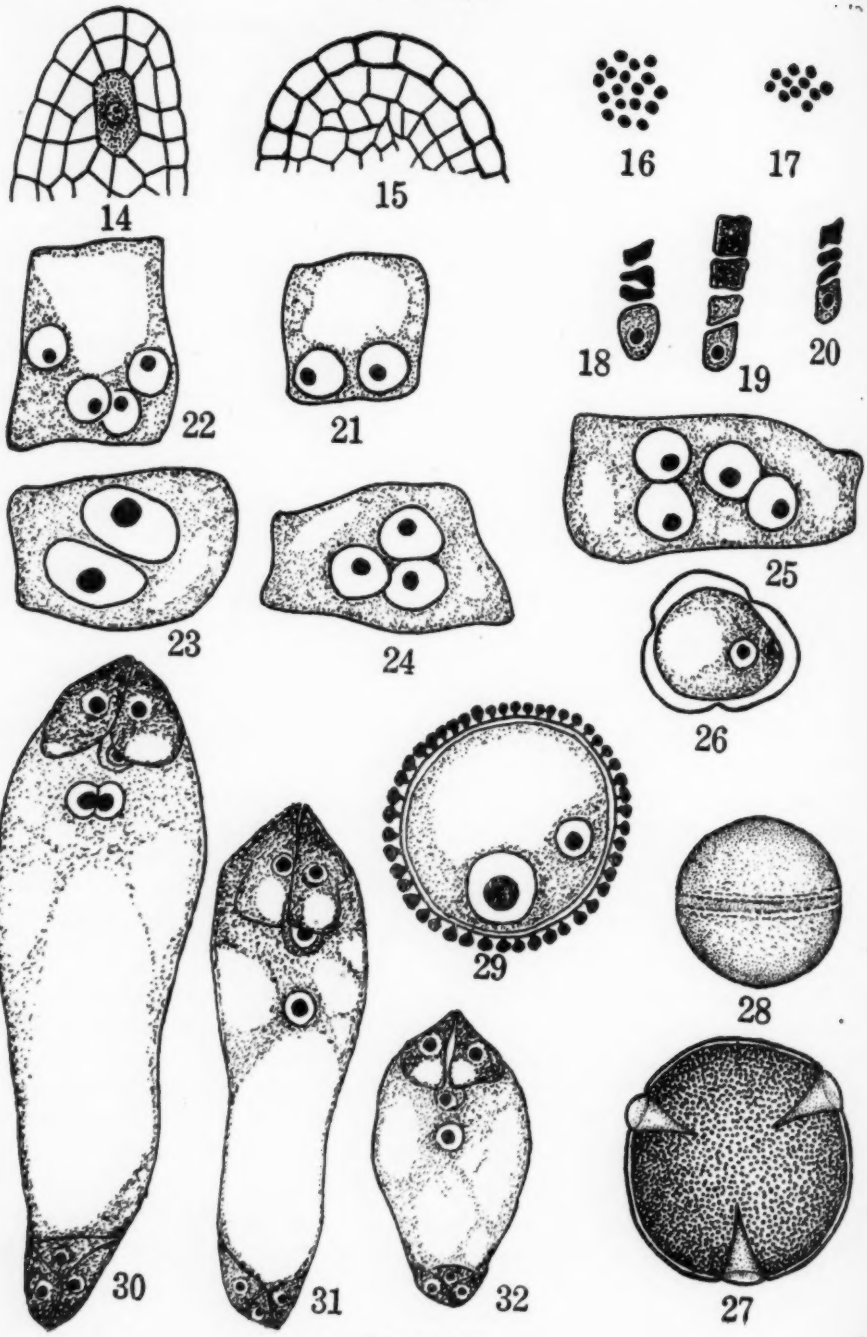

Figs. 14–32

TEXT-FIGS. 14–32.—Fig. 14. The origin of the M.M.C. in *P. tithymaloides*, × 250. Fig. 15. L. S. of part of a gland of *P. reticulatus*, × 250. Figs. 16 & 17. Haploid number of chromosomes in *P. tithymaloides* and *G. multiflorum*, × 900. Figs. 18, 19 & 20. Linear tetrad of megaspores of *G. multiflorum*, *P. tithymaloides* and *P. reticulatus* × 450. Figs. 21 & 22. Binucleate and quadrinucleate tapetal cells of *G. multiflorum*, × 900. Figs. 23, 24 & 25. Binucleate, trinucleate and quadrinucleate tapetal cells of *P. tithmaloides*, × 900. Figs. 26. Binucleate pollen grain of *P. tithymaloides*, × 250. Fig. 27. Outline of pollen grain of same showing three furrows (mounted in lactic acid), × 400. Fig. 28. Pollen grain of *P. reticulatus*, × 900. Fig. 29. A binucleate pollen grain of *G. multiflorum* × 900. Figs. 30, 31 & 32. Mature embryo-sacs of *P. tithymaloides*, *P. reticulatus* and *G. multiflorum*, × 900.

gradually attenuated at the apex, where it covers the nucellar tip and forms a narrow micropyle. The outer integument is massive, it stands apart from the inner, so that an open space is left between the two integuments. It completely covers the inner integument and forms a wide micropyle (Fig. 13).

The nucellus is massive and the beak is somewhat pointed. In *P. tithymaloides* the outer cells of the nucellar tip are elongated and form a well-defined layer as shown in Fig. 11. In *Phyllanthus reticulatus* the nucellus protrudes out of the integuments even at the megaspore mother-cell stage and later forms a beak-like structure which comes in contact with the cells of the obturator (Fig. 8).

The presence of the obturator could be detected at a very early stage of the development of the ovule in *P. tithymaloides* and *G. multiflorum* but not in *P. reticulatus*, where it develops comparatively later. In the first two plants mentioned, it is massive in structure and lies almost in contact with the integuments during the early stages of the development of the female gametophyte. In the mature ovule of the outer cells of the obturator in *P. tithymaloides* grow in the form of filaments and protrude inside the micropylar cavity (Fig. 11). In *G. multiflorum* it extends over tne top of the micropyle as a compact tissue, the outer cells of which grow as hairlike processes (Fig. 13). The obturator in *P. reticulatus* is composed of a few cells, the epidermal cells grow out in the form of short hairs and lie in contact with the nucellar beak (Fig. 8).

The vascular supply of the ovules in all the three plants terminate at the base of the nucellus at the chalazal region.

IV. *The development of the macrospores and the female gametophyte.*—In *P. tithymaloides* a single archesporial cell develops in the hypodermal layer of the nucellus. It cuts off a parietal cell and then functions as the megaspore mother-cell, which is therefore seen in the third layer (Fig. 14). In *G. multiflorum* the megaspore mother-cell was first observed in the fourth layer of the nucellus. The parietal cells overlying the megaspore mother-

cells undergo repeated divisions in both the plants, so that very soon the megaspore mother-cell is seen to become buried deep inside the nucellar tissue. The megaspore mother-cell increases in size and undergoes the normal reduction division. A linear tetrad of megaspores is produced in all the three plants (Figs. 18, 19 and 20). The disintegration of the megaspores takes place from above downwards. The functional megaspore by three successive divisions produces an eight nucleate gametophyte. The mature embryo-sacs are of the normal angiospermous type. The synergids are somewhat triangular in form in *P. tithymaloides* and *G. multiflorum*, with dense cytoplasm at the tips in which the nucleus is embedded. A vacuole occurs at the base. The egg lies below the synergids and occupies a more or less central position. The two polar nuclei generally lie close together in *P. tithymaloides* but they fuse in *G. multiflorum*. The antipodals are cut off as small cells and degenerate very early (Figs. 30 and 32).

The mature embryo-sac of *P. reticulatus* shows that the synergids are slightly hook-shaped. The egg lies below one of the synergids. The primary endosperm nucleus lies a little below the egg. The antipodals are three in number and degenerate early (Fig. 31). After the degeneration of the antipodals the embryo-sac elongates considerably in length.

Conclusion

Perry[12] has recently given a comprehensive account of the chromosome numbers of plants belonging to the family Euphorbiaceæ. He records 36 as the diploid number in *P. tithymaloides*, which corresponds with the haploid number 18 recorded in the course of the present investigation. The genus of *Gelonium* has not been studied previously and the haploid number 11 for *Gelonium multiflorum* appears to be the first to be recorded. The only other plant in the sub-tribe Gelonieæ whose chromosome number is known is *Baliospermum axillary* (*montana*), $2n = 28$. Incidentally it might be mentioned that Dutt[4] working in this laboratory first determined the haploid number of chromosomes as 20 for *Putranjiva Roxburghii* which Perry[12] later records as $2n = 14$.

The nature of the tapetal cells of only a few Euphorbiaceæ has been determined. Cooper[3] mentions the presence of binucleate cells in *Euphorbia splendens*, the division taking place at about the time of synizesis. Dutt[4] working on *Putranjiva Roxburghii* states that ordinarily spindle-shaped binucleate tapetal cells are formed most of which remain in that condition. In addition, quadrinucleate tapetal cells are formed as also tetraploid nuclei, due to fusion. As has been stated previously binucleate and quadrinucleate tapetal cells have been found both in *P. tithymaloides* and *G. multiflorum*.

In rare cases, trinucleate cells have been observed in the former plant. Critical examination of the tapetal cells of *Phyllanthus reticulatus* showed that the binucleate condition alone prevailed.

Another aspect of the tapetal cells which merits consideration, is their conspicuous radial elongation in the early meiotic stages of the pollen mother-cells of *Gelonium multiflorum*. The writer is not aware of similar condition prevailing in any other member of this family. Kajale and Rao's[7] sketch referred to as Fig. 29 depicting stages in the development of the anther in *Jatropha gossypifolia*, however, leads one to believe the existence of a similar condition in this plant.

Neither Wodehouse[15] nor Erdtman[5] has given any account of the morphology of the pollen-grains belonging to the family Euphorbiaceæ. Kajale and Rao[7] state that the pollen-grains of *Euphorbia hirta* possess a smooth surface and three broad furrows without any germ-pore and that of *Jatropha gossypifolia* is characterised by a thin membranous exine over which occur densely packed cone-like granules. The pointed ends being towards the centre of the grain. Further, the pollen-grains show complete absence of germinal furrows and germ-pores. It is interesting to recall that the pollen grains of *G. multiflorum* show an almost identical pattern of the exine and are further characterised by the absence of furrows and germ-pores. The pollen-grains of *P. tithymaloides* have already been described and until further information of the plants of the tribe Euphorbieæ is available they cannot be compared. The pollen-grains of *Phyllanthus reticulatus* are spherical in outline and show the presence of a central band inside which a single germ-pore appears to be present. The exine has a granulated surface (Fig. 28).

The development of the nucellus and obturator conforms to the types previously described. The nucellar beak observed in *Phyllanthus reticulatus* has also been recorded previously by Banerji and Dutt[2] in another species of *Phyllanthus*. The loose type of obturator prevails in *P. tithymaloides* and the compact form in the other two plants. The former type has been recorded by Kajale and Rao[7] in *Euphorbia hirta*, *E. prolifera* and *E. Wallachii* and also by Mc Cann[11] in the Tung oil tree. The latter type has been observed in *Jatropha gossypifolia*, *Putranjiva Roxburghii*, and other plants by different workers.

Embryosac formation in all the three plants is of the monosporic eight-nucleate type. The development of the female gametophyte in *P. tithymaloides* had been studied previously by Arnoldi.[1] The present observations confirm his findings.

Summary

The paper gives a comparative account of the development and morphology of the pollen grains, the ovule and embryo-sac of *Pedilanthes tithymaloides* and *Gelonium multiflorum*. A few observations on the aforesaid aspects of *Phyllanthus reticulatus* have also been incorporated.

The meiotic divisions in the pollen mother-cells of *P. tithymaloides* and *G. multiflorum* are normal. Eighteen haploid chromosomes have been observed in the former and eleven in the latter.

The tapetal cells are ordinarily binucleate. This condition is maintained in *P. reticulatus* but quadrinucleate cells have been noted not infrequently in *P. tithymaloides* and *G. multiflorum*.

The pollen grains of *P. tithymaloides* are triangular in form, tricolpate with three germ pores and granulated exine: those of *G. multiflorum* are without furrows and germ pores and show a characteristic pattern of the exine, while the pollen grains of *P. reticulatus* have a central band in the form of a ring inside which a single germ pore appears to be present. The exine shows a granulated surface.

The ovules are bitegmic. The nucellus projects greatly out of the micropyle in *P. reticulatus* only. The outer cells of the nucellar tip of *P. tithymaloides* are elongated.

The loose type of obturator has been noted in *P. tithymaloides* and the compact form in the other two plants.

The development of the female gametophyte corresponds to the normal eight nucleate type. The antipodals are ephemeral.

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THE INFLORESCENCE AND FLOWERS OF *DICHROSTACHYS CINEREA*, W & A

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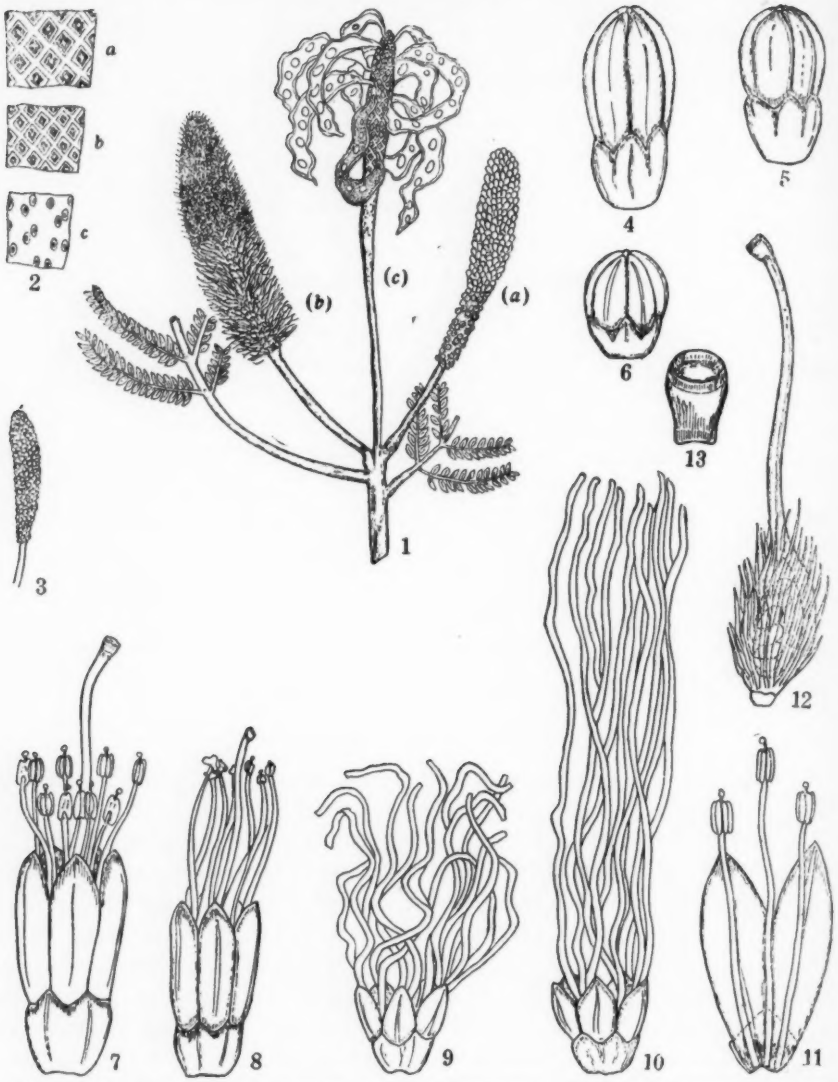
(Communicated by Prof. P. Maheshwari, F.A.SC.)

The inflorescences of certain members of the sub-family Mimosoideæ bear polygamous flowers. Geitler (1927) has described this phenomenon in *Neptunia oleracea*. The results of a similar study of *Dichrostachys cinerea*, another member of the sub-family, are embodied in the present paper. This plant is of frequent occurrence in the dry forests of India and material collected on the Delhi Ridge last September by Prof. P. Maheshwari was very kindly passed on to me for study.

Besides dissections of numerous flowers from several inflorescences, microtome sections of complete young spikes as well as of individual flowers were cut following the customary methods of infiltration and imbedding in paraffin. Some sections were stained with safranin and fast green and others with Heidenhain's iron-alum hæmatoxylin.

Observations

A portion of a flowering and fruiting twig of the plant is shown in Fig. 1. Exclusive of the stalk, approximately the upper half of the spicate inflorescence bears normal, perfect, bisexual flowers with the floral formula K (5), C (5), A 5, and *G* 1. The petals are subconnate towards their bases and are bright yellow in colour. The stamens have slender filiform filaments. The five stamens opposite the sepals are longer than those opposite the petals (Fig. 11). The dorsifixed anthers are four-celled, with the connective prolonged into a conspicuous, stipitate gland (Figs. 17, 18, 29). Four archesporial groups are differentiated in each anther (Fig. 30). The pollen grains in each anther locule are aggregated to form 6 or 7 pollinia arranged in a single file (Fig. 17). The ovary is one-celled and densely hairy (Fig. 12). The long style ends in a dilated, cup-like stigma with a marginal rim (Fig. 13). There are several ovules in two rows on a marginal placenta (Fig. 12). The anther filaments and the style are folded in bud but straighten out and become exserted in the open flower (Fig. 7).

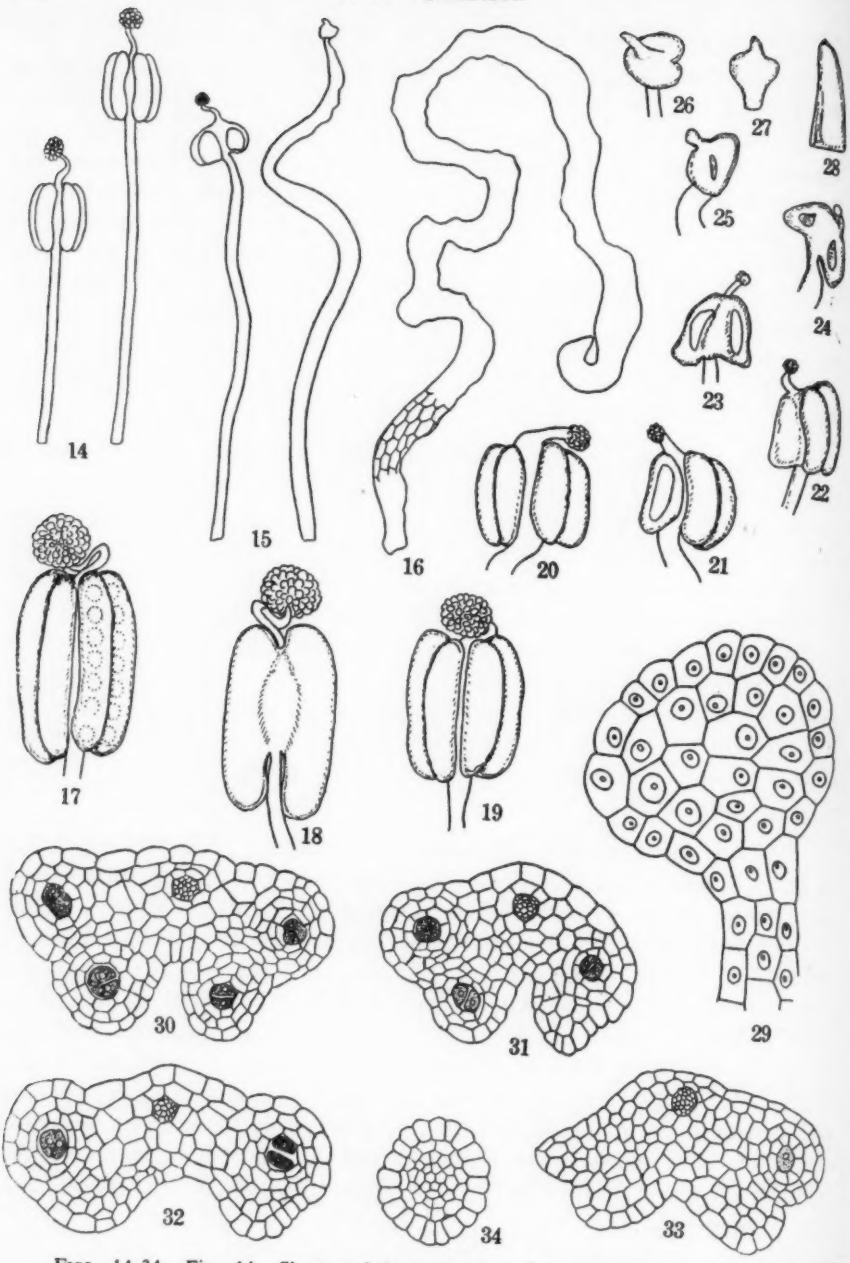


FIGS. 1–13. Fig. 1. Twig bearing three inflorescences with closed flowers (*a*), open flowers (*b*) and fruits (*c*), × 1. Fig. 2. Part of axis of inflorescence with flowers and bracts removed to show arrangement of scars of perfect flowers (*a*), flowers of intermediate zone (*b*), and sterile flowers (*c*). Fig. 3. Young spike showing compactly arranged flower buds, × 1½. Figs. 4–6. Buds of perfect (fig. 4), intermediate (Fig. 5) and sterile (Fig. 6) flowers, × 5. Figs. 7–9. Open flowers of three types, perfect (Fig. 7), intermediate (Fig. 8) and sterile (Fig. 9) × 5. Fig. 10. An open sterile flower with the tassel of staminodes straightened out, × 5. Fig. 11. Two petals and three stamens of a perfect flower, × 5. Fig. 12. Pistil of a perfect flower; dotted lines indicate position of ovules within, × 22½. Fig. 13. Enlarged stigma, × 70.

Flowers borne in the lower region of the inflorescence are sterile. The greatly shortened sepals and petals are somewhat pinkish in colour. Anthers are wanting and the bare filaments of the staminodes are greatly elongated and flattened and lie crumpled in bud. They form a dense tassel of tangled, thread-like, purplish-red structures which are much more conspicuous than the sepals or petals (Figs. 1 *b*, 9, 10). The tips of the staminodes show no differentiation into anthers (Figs. 28, 34). The pistillode presents a reduction series from one with a short style and stigma to a very minute hairy structure without any such differentiation. Ovules are either rudimentary or wanting. The reduction may be abrupt, belated or gradual so that the lowest flowers in an inflorescence may have only the barest rudiment of a pistillode, or a fairly large pistillode with a distinct style and stigma, or one with a very short beak-like style.

The demarcation between the upper fertile and lower sterile flowers is not so apparent in the youngest spikes (Fig. 3), but becomes more and more conspicuous in the later stages (Fig. 1, *a*) specially when the flowers open (Fig. 1, *b*). At first all the flowers are more or less of the same colour, but later the lower sterile flowers turn somewhat pinkish while the fertile upper flowers retain a bright yellow colouration. When in full bloom, the pretty nodding spike shows the bright yellow of the upper half in striking contrast to the red-purple of the lower half. All the flowers in the young spike are compactly arranged, but as it elongates the shorter flowers of the lower half appear to be more loosely arranged (Fig. 1, *a*). However, at the open-flower stage the lower zone again appears denser (Fig. 1, *b*) because of the tangled mass of staminodes.

Between the two zones described above are a few flowers which are of an intermediate nature. The calyx and corolla present a gradual reduction in size. A few such flowers immediately below the perfect flowers bear stamens with 4-celled anthers but these are slightly smaller than those of the perfect flowers (Figs. 19, 20). Flowers still lower bear very small, variously deformed rudimentary anthers with 3, 2 or 1 loculus only (Figs. 21–25, 31–33). The terminal gland also shows corresponding reduction from a conspicuous stipitate structure to just a blunt, shortened stump (Figs. 25–27). The filaments show a marked tendency to elongate, broaden and twist like the staminodes (Fig. 15). The gynœcium in these flowers is almost normal except for a slight decrease in size. Further down the inflorescence are a few flowers that are apparently female with the stamens devoid of anthers.



FIGS. 14–34. Fig. 14. Short and long stamens of a perfect flower, × 5. Fig. 15. Short and long stamens of an intermediate flower, × 5. Fig. 16. Staminode from

sterile flower, × 5. Figs. 17–18. Ventral and dorsal views of anther from a perfect flower (dotted lines indicate position of the pollinia within), × 45. Figs. 19–24. Anthers from intermediate flowers with all four loculi intact (figs. 19, 20), with one loculus reduced (Fig. 21), and with two loculi reduced (Figs. 22–24), × 45. Figs. 25–27. Other deformed anthers, × 45. Fig. 28. Tip of a staminode showing lack of anther, × 45. Fig. 29. L.S. anther gland from a perfect flower, × 730. Fig. 30. T.S. perfect anther with 4 archesporial groups, × 320. Figs. 31–33. T.S. anthers with 3, 2 and only 1 archesporial group respectively, × 320. Fig. 34. T.S. tip of staminode showing lack of differentiation of anther, × 320.

Of the total number of flowers in a spike, the perfect flowers are the most numerous, the sterile flowers fewer and the intermediate flowers fewest. Counts made on four spikes gave the average of 117 fertile, 34 sterile and 11 intermediate flowers.

As expected, in the fruiting spike the twisted pods are confined only to the upper zone (Fig. 1 *c*).

It is clear from the above account that sterilization in the inflorescence is in the basipetal direction. In an individual flower it is centripetal, progressing from the stamens to the pistil. Sterilization in both cases is gradual, the perfect flowers in the spike being separated from the sterile flowers by a few transitional types.

Summary

The inflorescence of *Dichrostachys cinerea* is described in detail. In addition to the two externally well-marked zones consisting of the upper perfect and bisexual flowers, and the lower sterile ones, there is a short intermediate zone of a few flowers which form a transition between the two. Sterilization of the inflorescence is basipetal; of the flower centripetal.

The author expresses his grateful thanks to Prof. P. Maheshwari, for material and guidance and to Dr. B. M. Johri for helpful suggestions.

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EMBRYOLOGICAL STUDIES IN THE LEGUMINOSÆ

III. A Contribution to the Embryology of *Pithecolobium saman* Benth. Syn. *Enterolobium saman* Prain

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Introduction

For some time past, local members of the Mimosaceæ have been under investigation with the object of studying their embryology. The work on two plants has already been completed (Dnyansagar, 1949, 1950). The present contribution is the third in the series. In *Leucæna glauca* and *Mimosa hamata*, the plants already worked out by the author (Dnyansagar, 1949, 1950), a number of common features, such as a single-layered tapetum of the secretory type, a massive nucellus, extensive parietal tissue during megasporogenesis, the inner integument developing first and the outer overgrowing it, hooked synergids with filiform apparatus, the division of the primary endosperm nucleus before the first segmentation of the oospore and lastly, a massive proembryo without any suspensor, were observed. These, it is interesting to record, have also been found in *Pithecolobium saman*. In addition, the present plant resembles *Mimosa hamata* in hypodermal origin of the archesporium and *Leucæna glauca* in regard to the formation of the micropyle by the outer integument alone. But it differs from both the other plants in the comparatively lesser number of microspore mother-cells and the formation of pollinia.

The relevant literature has been quoted previously and it is hardly necessary to repeat the same.

Material and Methods

Material was collected from the plants growing in Nagpur and the Botanic Gardens, Poona. The number of fruits formed in the case of trees growing in Poona was several times more than in the case of the trees growing in Nagpur; so most of the fruit-collections were made from the trees growing in Poona. The material was fixed in the formalin-acetic-alcohol and Randolph's modification of Navaschin's fluid (Johansen, 1940). Sections were cut 8–12 μ thick and were stained chiefly with iron-alum hæmatoxylin and differentiated in an aqueous solution of picric acid (Maheshwari,

1933). Earlich's hæmatoxylin (Johansen, 1940) was also used. Both the stains were found to be satisfactory. For root tips, iron-alum hæmatoxylin and Feulgen's stain were used. For pollen preparation, methyl green-glycerene jelly (Wodehouse, 1935) and anilin-oil-gentian violet method (Wodehouse, 1935) were employed.

Chromosome Number

Chromosome counts were made from polar views of the metaphase in the cells of the root-tip. The somatic or 2 *n*-chromosome number was determined to be 18 (Fig. 2). Darlington and Janaki Ammal (1945) have given the list of somatic or diploid number of chromosomes as 24, 26, 48 and 52 in the Mimosaceæ. The number 18 is, therefore, an addition to the list.

The Inflorescence and the Flower

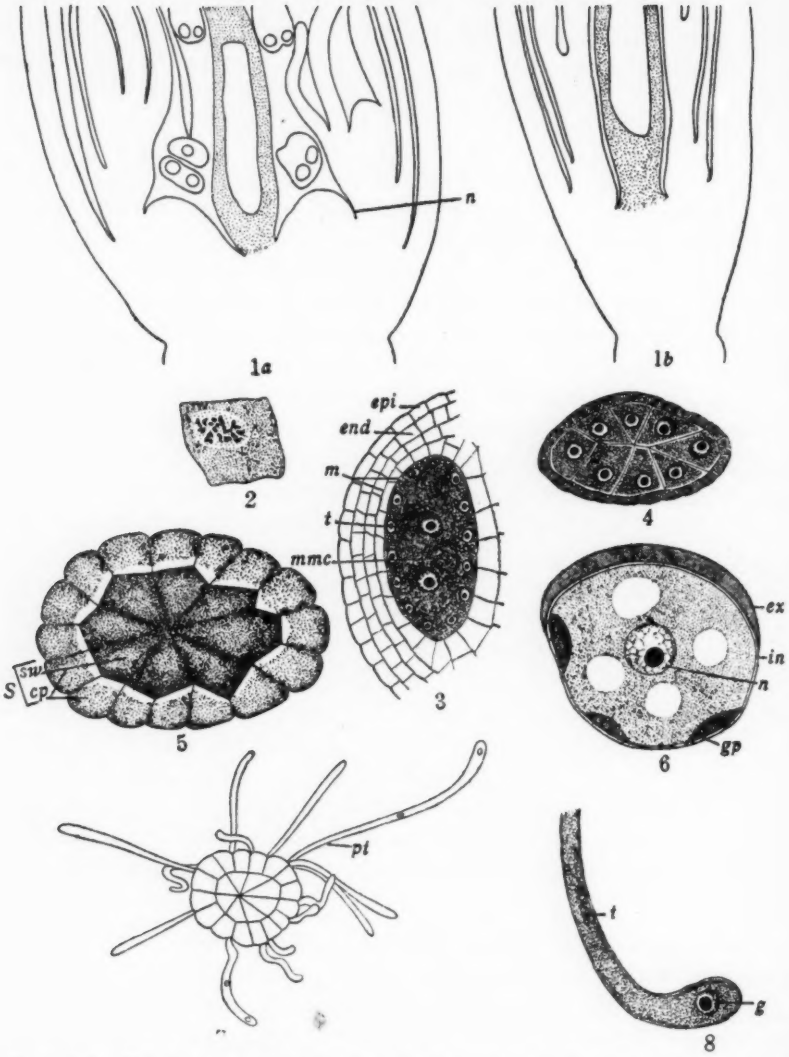
Pithecolobium saman, popularly known as Rain tree, is a big tree of rapid growth with large spreading branches. It looks beautiful with its numerous rose-coloured heads of flowers with long stamens against the dark green leaflets. The inflorescence is an axillary globose head with bisexual flowers. The latter are rose-coloured and range from 15–25 per inflorescence. In each head, the terminal or central flower is distinct from the others as in *Albizzia lebbek* (Maheshwari, 1931). It is larger in size and swollen at the base due to the presence of a nectary (Fig. 1 *a*) which does not develop in the lateral flowers (Fig. 2 *b*). Occasionally, two carpels, separate from each other, are found in a flower.

The flowering period commences from April and lasts upto July. Most of the inflorescences drop off especially during summer and only a few are left to form fruits which begin to mature in January. The number of fruits in each inflorescence is very small being only one or two. It is usually from the lateral flowers that the fruits are formed as in *Albizzia lebbek* (Maheshwari, 1931).

The floral parts arise in acropetal succession and are cyclic in their arrangement as in *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa hamata* (Dnyansagar, 1950). The floral formula is $K_{(5)}$, $C_{(5)}$, $A\ \infty$, G-*l*. The androœcium is monoadelphous and the filaments are long and pink.

Microsporogenesis

The wall of the young anther beneath the epidermis is made up of the endothecial layer, one or two middle layers and a unilayered tapetum (Fig. 3). The tapetum is of the secretory type and its cells remain uninucleate throughout as is the case in all the investigated species of the Mimosaceæ.



FIGS. 1–8. Fig. 1 A. Longitudinal section of basal part of terminal flower showing nectary, *n* at the base. B. Longitudinal section of basal part of lateral flower with no nectary, × 2·5. Fig. 2. Polar view of metaphase in mitotic division in a cell of root tip showing 18 chromosomes, × 950. Fig. 3. Transverse section through part of an anther showing microspore mother-cells, *mmc*, tapetum of uninucleate cells, *t*, middle layers, *m*, endothecium, *end.* and epidermis *epi.*, × 500. Fig. 4. Transverse section of part of an anther showing microspores surrounded by uni-layered tapetum, × 225. Fig. 5. Surface

view of 32-celled pollinium showing 16 peripheral and 16 central grains, the latter being in 8 superposed pairs. The pollinium consists of 8 sectors, each sector, *s* being made up of 2 peripheral, conjoint and polyhedral grains, *cp* and 2 central, superposed and wedge-shaped grains, *sw*, × 225. Fig. 6. Individual pollen grain showing exine, *ex*, intine, *in*, 3 germ-pores, *gp* and nucleus, *n*. × 1,000. Fig. 7. Pollinium showing pollen tubes, *pt* emerging from its grains, × 100. Fig. 8. Part of pollen tube showing generative cell, *g* and tube-cell, *t*, × 1,000.

The wall of the mature anther consists of the epidermis and the endothecium which is now prominent with fibrous thickened bands. At this stage, the tapetum and the middle layers can no longer be seen having been used up during sporogenesis. In each microsporangium, usually 8 microspore mother-cells are produced, these after undergoing the reduction divisions, produce 32 microspores which adhere together to form a pollinium (Fig. 4 and Plate VII, Fig. 1) as in *Albizzia lebbek* (Maheshwari, 1931), *Acacia Baileyana* (Newman, 1934) and *Acacia farnesiana* (Narasimhachar, 1948).

A mature pollinium is 120–35 μ in diameter. It consists of generally 32 grains and occasionally 28 grains. In the normal 32-celled pollinium which takes up a somewhat flattened elliptical or spherical shape, 16 grains are arranged centrally and 16 at the periphery, those in the centre being in two superposed groups of 8 each. The 16 peripheral grains are all in a plane at right angles to and bisecting the central group. The peripheral grains are so placed that their 16 contacts with each other are alternately opposite and midway between 8 contacts of the central group. As a result of such arrangement, the group as a whole has become flattened with the intersecting lines between the individual grains crossing each other at right angles.

Fig. 5 shows a normal elliptical 32-celled pollinium consisting of 16 peripheral grains and 16 central grains in two superposed groups of 8 each. It is made up of 8 tetrahedral tetrads each occupying a sector; in each sector, two grains are at the periphery and these are conjoint and polyhedral (*cp*) and two are at the centre, these being superposed and wedge-shaped (*ws*) as indicated in one such sector, *s*.

The individual grains are 25–30 μ in diameter. In each grain, the exine on the exposed surface is thick while that on the inner side is thin and delicate, allowing the grain to be suitably shaped by the pressure against the neighbouring cells. Underneath the intine, there are three small areas placed at three corners opposite to the thickened exine (Fig. 6). These are the germ pores.

The pollinia are shed as such. Each grain has vacuolated cytoplasm and contains a single nucleus in a young pollinium. It becomes bi-celled at the time of its shedding.

Germination of the Pollen-Grains

The germination of the grains has been studied by examination of both fixed carpels and living material in culture. A large number of styles were dissected after maceration in 40% acetic acid at 45° C. Pollinia were found to be lodged in the stigma.

60–70 pollinia were dusted on 15%, 20% and 30% cane-sugar solutions to which neutral red was added for vital staining. A few pollen-grains in two or three pollinia germinated and sent out the tubes. Brough (1927) used this method of culture on the surface employing cane-sugar solutions of various concentrations successfully with *Dampiera stricta*. Newman (1934), however, failed to get positive results by this method with *Acacia Baileyana*.

Pollinia were also dusted on slides with a thin film of sugar-agar using 1% agar and 15%, 20% and 30% concentrations of cane-sugar. The medium with the sugar-agar ratio 20 : 1 gave satisfactory results. 35% to 45% grains in a pollinium germinated. The tubes attained a length of 0·10 mm. to 0·15 mm. The pollen-tubes were formed from the inner sides of the grains and emerged between the individual grains (Fig. 7) as in *Acacia Baileyana* (Newman, 1934) and other species of *Acacia* (Wodehouse, 1935).

It appears that the wall of the pollen-tube is a continuation of the intine. The generative cell precedes the tube cell into the tube (Fig. 8) as in *Acacia Baileyana* (Newman, 1934). The tube nucleus subsequently disorganises. A spindle of dividing generative nucleus parallel to the shorter axis of the tube was seen in the case of a tube grown on sugar solution.

Megasporogenesis

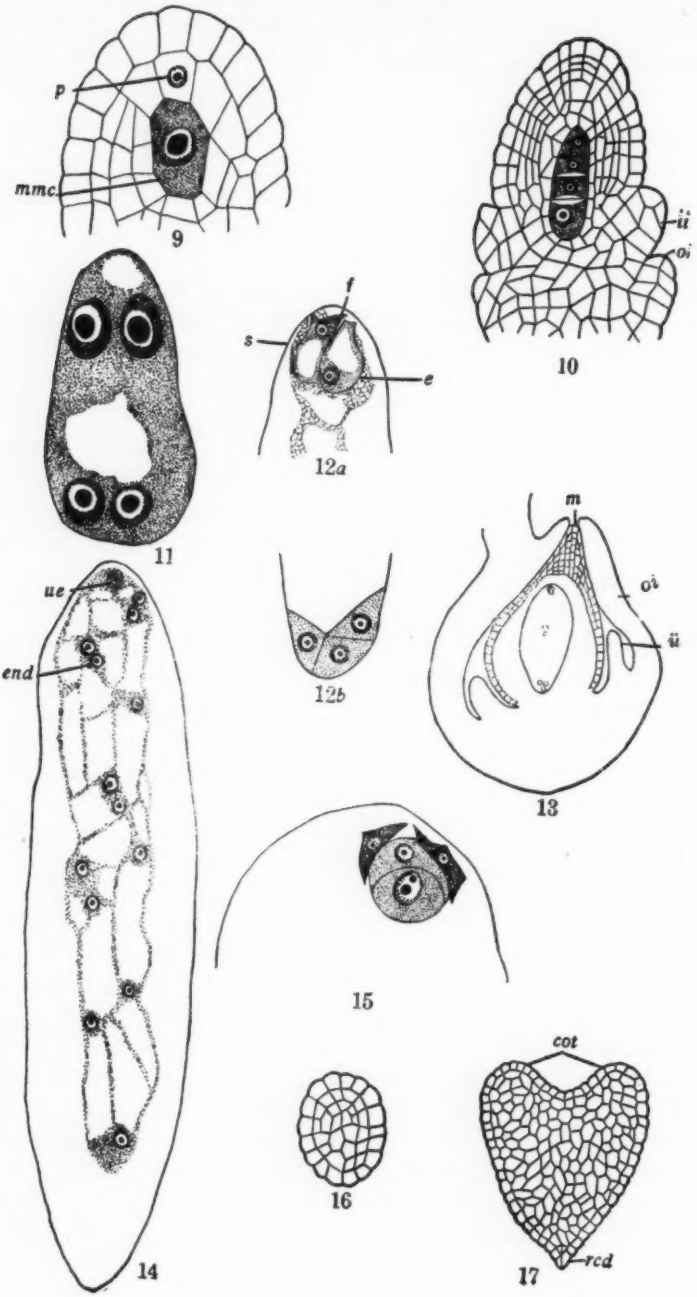
6-15 Ovules are borne in two alternating rows on the ventral suture of the ovary. These are seen as small papillæ at the time, microspore mother-cells have been formed in the anthers. The young ovules are at first quite straight, standing almost at right angles to the ventral suture but they begin to curve upwards towards the stylar end on coming close to the dorsal wall of the ovary and assume an anatropous form. This gradual assumption of anatropy has been reported in *Albizzia lebbek* (Maheshwari, 1931), *Neptunia oleracea* (Singh and Shivapuri, 1935), *Leucæna glauca* (Dnyansagar, 1949), *Mimosa hamata* (Dnyansagar, 1950) and several other leguminous plants.

Each ovule shows a massive nucellus right from the start, as in *Albizzia lebbek* (Maheshwari, 1931), *Neptunia oleracea* (Singh and Shivapuri, 1935), *Cassia* (Pantalu, 1945), *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa*

hamata (Dnyansagar, 1950). But at the time of formation of the tetrad of megaspores, there are 5–6 layers of the nucellar cells above the tetrad, 5–6 layers on the sides and 3–5 layers below (Fig. 10).

The two integuments begin to appear after the archesporium has been differentiated, the inner one appearing first and the outer one almost immediately afterwards. Both the integuments, at this stage, consist of two layers of cells each (Fig. 10), as in *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa hamata* (Dnyansagar, 1950). Later, the outer integument begins to grow faster and ultimately alone forms the micropyle (Fig. 13), as in *Albizzia lebbek* (Maheshwari, 1931), *Neptunia oleracea* (Singh and Shivapuri, 1935) and *Leucæna glauca* (Dnyansagar, 1949); it now consists of several layers of cells in the region surrounding the micropyle and gradually thins out towards the base. The further development of the inner integument after its initiation is perhaps unlike anything so far recorded in the previously investigated species of the Mimosaceæ. It reaches at the most, the middle of the nucellus when the embryo-sac is fully mature and the micropyle completely organised at the top (Fig. 13). And what is still more interesting is that this integument starts developing again after fertilisation and ultimately reaches the upper level of the nucellus when the endosperm is just beginning to be formed (Plate I, Fig. 2).

The archesporium is hypodermal and consists of a single cell. It differentiates even before the appearance of the integument primordia. Such an early differentiation is a common occurrence in the Leguminosæ. The archesporial cell cuts off a parietal cell (Fig. 9) which divides both anticlinally and particularly forming an extensive parietal tissue as a result of which the megaspore mother-cell becomes deep-seated. Such an extensive development of the perietal tissue seems to be characteristic of the Cæsalpiniaceæ and Mimosaceæ, as has been stated by Pantalu (1945). About 5–6 epidermal cells at the micropylar end divide periclinally and form a distinct epidermal cap over the parietal tissue (Fig. 10). Such a multiple epidermal cap has been recorded by Guignard (1881) as a common feature in the Leguminosæ. It may further be mentioned that some epidermal cells on lateral sides of the ovule, become involved in the process and ultimately a very much extensive cap is seen at the time of the mature embryo-sac (Fig. 13). The megaspore mother-cell, after its differentiation, enlarges considerably and in doing so, it crushes the surrounding nucellar cells. After a period of rest, it undergoes two reduction divisions and a linear tetrad of megaspores is formed. The lowermost develops into the normal type of the embryo-sac after three successive free nuclear divisions (Figs. 11 and 12).



FIGS. 9–17

FIGS. 9–17. Fig. 9. Longitudinal section of nucellus showing parietal cell, *p* which has divided anticlinally and megaspore mother-cell, *mmc.*, × 1,000. Fig. 10. Longitudinal section of ovule showing deep-seated linear tetrad of megaspores and primordia of two integuments, *oi* and *ii.*, × 450. Fig. 11. 4-Nucleate stage of embryo-sac, × 1,000. Fig. 12. Mature embryo-sac showing (*a*) egg apparatus consisting of an egg, *e* and two synergids, *s*. Fig. 12 *a*. Micropylar part of embryo-sac showing egg-apparatus. One synergid, *s* with filiform apparatus, *f* and egg, *e* are seen in this section. 12 *b*, chalazal part of embryo-sac showing three antipodal cells. Fig. 13. Longitudinal section of ovule with its inner and outer integuments, *ii* and *oi* respectively, micropyle, *m* and epidermal cap, *epc*, × 225. Fig. 14. Embryo-sac showing undivided oospore, *ue* and free endosperm nuclei, *end*, × 225. Fig. 15. Upper part of embryo-sac showing transversely divided oospore, *e* and remnants of two disorganised synergids, *s*, × 500. Fig. 16. Massive proembryo, × 225. Fig. 17. Advanced pro-embryo showing tip of radicle, *rcd* and two cotyledons, *cot* × 100.

The two synergids are hooked and have each a filliform apparatus at the apex (Fig. 12 *a*). Dahlgren reviewed the literature on hooked synergids in 1938. He states that among the Mimosaceæ they have been reported in *Albizzia lebbek*, *Acacia Baileyana* and *Neputunia oleracea*. To this list, may be added *Acacia farnesiana* (Narasimhachar, 1948), *Leucæna glauca* (Dnyansagar, 1949), *Mimosa hamata* (Dnyansagar, 1950) and *Pithecolobium saman*. Among the Papilionaceæ and Cæsalpiniaceæ, these have been reported only in *Medicago sativa* (Cooper, 1935) and *Cassia* (Pantalu, 1945) respectively. The antipodals are definite cells (Fig. 12 *b*). The two polar nuclei meet more or less near the centre.

Starch grains are deposited in the embryo-sac at the 8-nucleate stage. The presence of starch in the embryo-sac seems to be of wide and variable occurrence. Dahlgren (1939) has reviewed the literature on the presence of starch grains in the embryo-sacs. According to him, in general, the production of starch grains seems to attain its maximum immediately before fertilisation. The present observation agrees with his statement. Among the Mimosaceæ, these have been reported to occur from the 8-nucleate stage of the sac onwards in *Albizzia lebbek* (Maheshwari, 1931), *Neptunia oleracea* (Singh and Shivapuri, 1935), *Acacia farnesiana* (Narasimhachar, 1948) and *Mimosa hamata* (Dnyansagar, 1950). In *Acacia Baileyana* and *Acacia discolour* (Newman, 1934), starch appears in the early stages of the germination of the megaspore. Among the Papilionaceæ, it is found by Reeves (1930), Cooper (1935), Cooper, Brink and Albrecht (1937) in *Medicago sativa* from the 2-nucleate stage and it is abundant in mature embryo-sacs.

ENDOSPERM AND EMBRYO

The primary endosperm nucleus divides rapidly by many free nuclear divisions before the first segmentation of the oospore as in *Acacia farnesiana* (Narasimhachar, 1948), *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa*

hamata (Dnyansagar, 1950). Due to these divisions, the sac becomes filled with endosperm nuclei and at the same time, it enlarges resulting in the appearance of vacuoles here and there (Fig. 14). Cell formation commences at the micropylar end and proceeds along the wall of the sac, resulting in the formation of a distinct parietal layer all round. It then advances from the micropylar side downwards till just a part is left on the chalazal side in which only free endosperm nuclei are present, the walls being not laid even as late as the appearance of the cotyledons in the embryo (Plate VII, Fig. 3). Similar condition was observed by Narasimhachar (1948) in *Acacia farnesiana*. No trace of the endosperm was observed in the mature seed of the *Pithecolobium saman*.

The first division of the oospore is transverse (Fig. 15, Plate VII, Fig. 2), as in *Acacia Baileyana* (Newman, 1934), *Acacia farnesiana* (Narasimhachar, 1948), *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa hamata* (Dnyansagar, 1950). In the preparation illustrating this division, the disorganised synergids were seen lying in their respective positions one on either side, even the remains of the filiform apparatus being slightly visible in one of the synergids. It could not be determined whether the second wall was transverse or longitudinal. There, however, seems to be no doubt that the further divisions occur in all planes and a massive proembryo is formed (Fig. 16). It is usually spherical, sometimes it assumes various forms of pear shape. It has no suspensor. Such a massive type of the proembryo has been described in several species of the Mimosaceæ, *e.g.*, *Acacia Baileyana* (Newman, 1934), *Acacia farnesiana* (Narasimhachar, 1948), *Leucæna glauca* (Dnyansagar, 1949) and *Mimosa hamata* (Dnyansagar, 1950). Fig. 17 shows the embryo consisting of young radicle and two cotyledons between which there is the region whence the plumule will arise.

Summary

1. *Pithecolobium saman* Benth. is a large tree of rapid growth with large spreading branches.

2. The inflorescence is a globose head and consists of 15–25 flowers. The central flower in each head differs from other flowers in the presence of a nectary which is lacking from others. The number of fruits that are formed per inflorescence is 1–2.

3. The $2n$-chromosome number is 18.

4. During microsporogenesis, 16–32 megaspores are produced per microsporangium and these form a single pollinium.

5. Pollinia are shed as such and individual pollen-grains are bi-celled at the time of shedding. The pollen-tubes are formed from the inner side of the grains and emerge from within the group between the individual grains.

6. The ovules are anatropous and have two integuments, of which the outer forms the micropyle.

7. Megasporogenesis is of the normal type. The archesporium is single-celled and hypodermal. Extensive parietal tissue is formed as a result of several periclinal and anticlinal divisions of the primary parietal cell. A linear tatrad of megaspores is formed, of which the lowermost functions.

8. The embryo-sac conforms to the 8-nucleate Normal type. The synergids are hooked and each has a filiform apparatus. The antipodals form definite cells.

9. The primary endosperm nucleus divides several times before the first segmentation of the oospore. Wall formation in the endosperm commences from the micropylar end, the chalazal end containing only free endosperm nuclei for a long period.

10. The first division in the oospore is transverse. Later, by repeated divisions, a spherical or pear-shaped proembryo is formed. This latter has no suspensor.

In conclusion, the author wishes to express his gratitude to Prof. R. L. Nirula, under whose guidance the work was carried out for helpful suggestions and criticism throughout the course of this investigation.

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Explanation of Plate

Plate VII

Fig. 1. Transverse section of part of anther showing pollen grains surrounded by parietal tissue, × 525.

Fig. 2. Upper part of the sac showing the transversely divided oospore, *e* remnants of two disorganised synergids, *s*, free endosperm nuclei, *end* and the inner integument, *ii*, ×140.

Fig. 3. Longitudinal section of older seed showing embryo with two cotyladous and cellular endosperm which has not extended upto the chalazal end, × 46.

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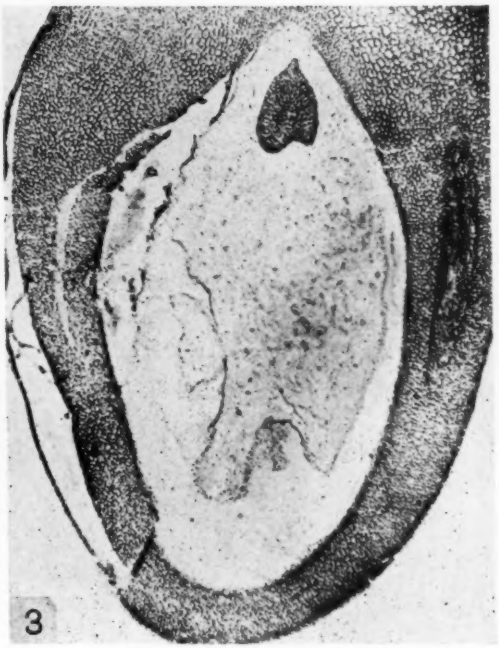
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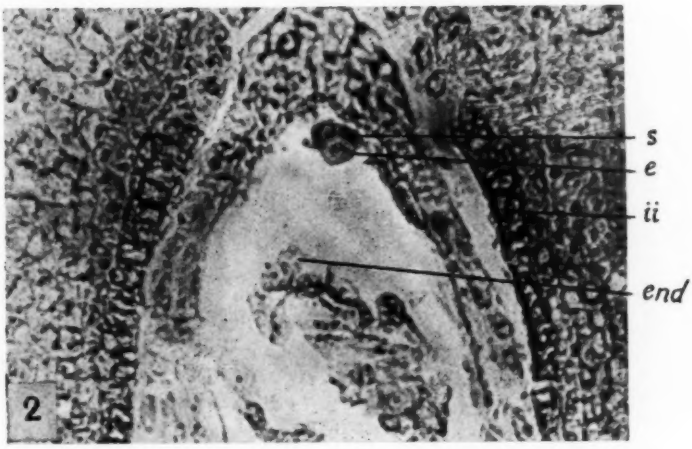
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STUDIES ON THE OSMOTIC PROPERTIES OF THE EGGS AND LARVÆ OF A BRACKISH-WATER POLYCHÆTE, *MARPHYSA GRAVELYI* SOUTHERN

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Introduction

While the osmotic behaviour of marine polychætes has been studied by several authors (Schlieper, 1929; Ellis, 1933, 1937; Beadle, 1934, 1937, 1943; Bethe, 1934; and Adolph, 1936), the osmotic properties of their eggs and larvæ have received no attention. McClendon (1910 *a*, *b*), R. S. Lillie (1916, 18), Runnstrom (1925), Luke and McCutcheon (1925–26), Bialaszewicz (1927), Needham (1930), Euphrussi and Rapkine (1928), Needham and Needham (1930 *a*, *b*), Ranzi (1930) and Northrop (1926–27) have performed experiments on the osmotic properties of tne eggs of sea urchins and of Sepia among the invertebrates, but have not investigated the behaviour of the larvæ and adults of these forms. An investigation of the osmotic properties of the eggs, of larvæ and of the adult of the same species may throw light on the adaptations of species to different habitats during their whole life cycle. In the present paper an account of the behaviour of eggs and larvæ of the brackish water polychæte, *Marphysa gravelyi* Southern is given and will be followed later by a study of the osmotic regulations of the adult.

Material and Methods

North of the Zoology Laboratory (Madras University) where the present investigations were carried on is the River Cooum. Except in the monsoons the river does not flow into the sea because of the formation of a sand bar at its mouth. By virtue of the formation of the sand bar at the mouth, about 100 yards up the river, the bed of the river is sandy and beyond it, it becomes gradually soft and clayey. The salinity of the waters at the mouth is almost that of seawater and decreases as one travels up the river into the interior. In the vicinity of the clayey region the salinity of the water ranges between 20 $^{o}/_{oo}$ and 34 $^{o}/_{oo}$ in the different months of the year, it being the minimum during the rainy season and highest in the hotter months. Thus there are fluctuations in the degrees of salinity all the year round. Here

may be seen during February to September, and in lesser numbers in the months of December to February (Aiyar, 1931), numerous pear-shaped masses of jelly which are the egg-cases of *Marphysa gravelyi*. Embedded in this jelly are large numbers of eggs, distributed evenly throughout the spawn. These egg-cases are firmly rooted in the soft mud by long stalks. Since the soil is soft, they can, however, be dug out easily. Such egg-cases were collected carefully and brought to the Laboratory along with the water. They were left in glass tanks and samples of eggs were removed from the cocoon from time to time for study. An account of the development has already been given by Aiyar (1931). As an adaptation to its habitat, the *trochophore* stage in the development of *Marphysa* is eliminated and even the earlier stages of development are undergone within the jelly. It is only when a stage which corresponds to the initial *metatrochophore* stage is reached, after a period of 3½ days, that the larvæ come out of the jelly and swim about in the medium. Such larvæ were collected for experimentation.

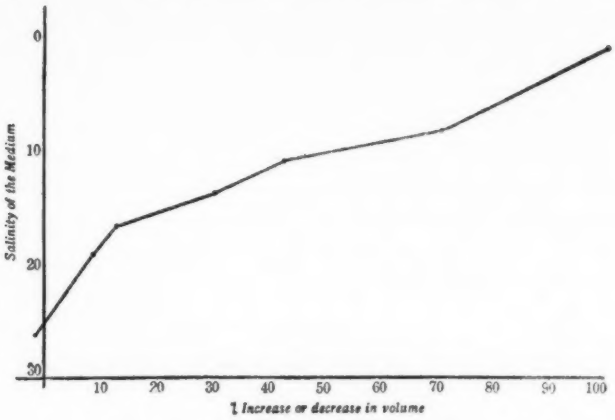
The object of the present investigation being the determination of the effects of different concentrations on the volume changes in the eggs and larvæ, it was necessary to measure their volumes. The method suggested by Weil and Pantin (1931 *a*, *b*) was followed in determining the diameter and area of the eggs and larvæ respectively. The diameter was measured direct by making use of an ordinary ocular-micrometer. Instead of a ghost-micrometer a net-micrometer was used in the eye-piece and the areas of the larvæ were directly read off. For every such determination eye-piece $\times 5$ and objective $\times 40$ were used and the magnification kept constant. Since the eggs are spherical, the volumes of the eggs were calculated by making use of the formula $1/6\ 22/7\ d^3$ as suggested by Krogh (1939). The volume of each egg and larva recorded in this paper represents the mean volume of six readings. For experimentation the eggs and larvæ from a single spawn were used.

A. *Experiments on Eggs*

EXPERIMENT I.—*Hypotonic Media and its Effect on Volume Changes in the Eggs*

(*a*) *Increase in Volume in Different Hypotonic Media.*—Six eggs of a single spawn were isolated from the jelly and the diameter was first measured and the volume calculated. They were transferred to petri dishes containing sea water of a particular strength for 30 minutes. At the end of which the diameter of each egg was read and the volume calculated.

In a similar way the experiment was repeated with different concentrations of the medium. Graph 1 indicates that the increase in volume is



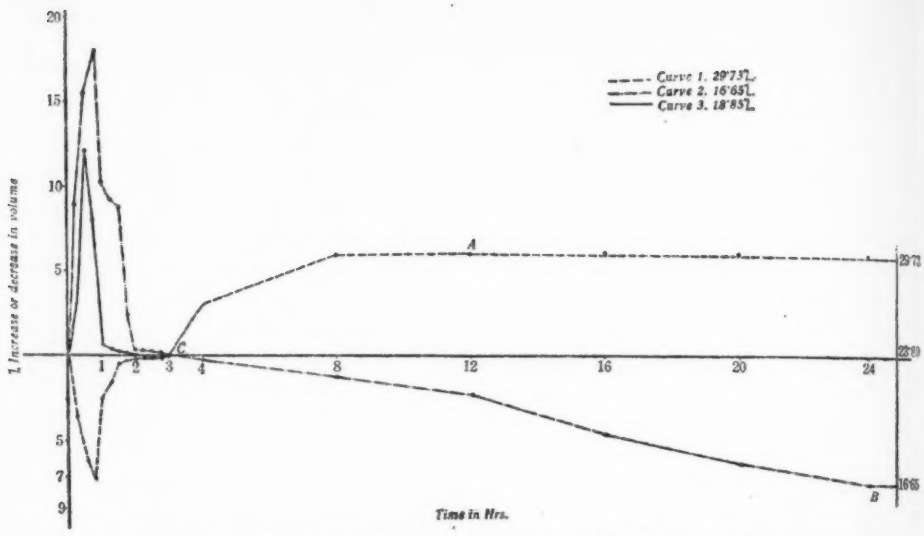
GRAPH 1. Increas in volume in different Hypotonic Media

proportional to the dilution of the medium used. This observation agrees with the results of Lillie (1916) and Runnstrom (1925) on the eggs of *Arbacia* and *Paracentrotus lividus* respectively.

(*b*) *Volume Changes in Hypotonic Media during Different Intervals.*— 400 Eggs taken from a single spawn were left in a petri-dish containing hypotonic medium of a known concentration. The volumes of six eggs were determined for a period of 3½ hours at intervals of every 15 minutes. The figures recorded indicate that the volume increases in about 30 to 45 minutes and decreases subsequently (Curve 3, Graph 2). The eggs reach their original volumes irrespective of the strength of the medium within 3½ hours.

(*c*) *Volume Changes in Hypotonic Media during Different Intervals.*— Five to six hundred eggs were immersed in a medium of a particular concentration (16·65‰) and the volumes of six eggs were determined after an interval of every 15 minutes for a period of 3 hours and later after intervals of 4 hours for a period of 40 hours. Curve 2 in Graph (2) shows the results of such an experiment. They indicate that the eggs increase in volume within the first half hour and then begin to decrease in volume. The volume shrinks to the original level in 3½ hours but the decrease continues for about 24 hours till the egg is 92·6% of the original size. There is no shrinkage beyond this upto a period of 40 hours.

During this decrease in volume obviously due to loss of salts, nearly 41·2% of the eggs died within 24 hours and about 50% died in 40 hours. It is quite probable that the initial increase in volume was due to the higher concentration of the egg and later due to loss of salts as well as water there



GRAPH 2. Volume Changes in Hypotonic and Hypertonic Media during different intervals

was shrinkage of volume. The differences in the rate and amounts in loss of salts essential for normal well being, probably account for the mortality of 50% of the eggs—for by 96 hours all the eggs died (*vide infra*).

(*d*) *Volume Changes in Hypertonic Media during Different Intervals.*—200 Eggs were immersed in a hypertonic medium of 29·73‰ and the volumes of six eggs were determined after an interval of every 15 minutes for a period of 3 hours and later after intervals of 4 hours for a period of 32 hours. Curve 1, Graph (2) shows the results of such an experiment. The readings recorded indicate that the volumes decrease at first and reach the maximum in 45 minutes and subsequently the volumes increase. The volume increases to the original level in 3 hours and the increase continues for about 12 hours till the egg is 106·2% of the original size. There is no increase in volume beyond this upto a period of 32 hours.

During this increase in volume, obviously due to uptake of salts and water, nearly 41·6% of the eggs died within 12 hours and about 75% died in 32 hours. It is quite probable that due to the higher concentration of the external medium, there is an initial decrease in volume and later due to uptake of water as well as salts from the medium there is an increase in volume. The differences in the rates of diffusion of salts and water into the egg probably account for the different percentages of mortality and also the final volume being greater than the original.

A comparison of the Curves 1 and 2 in Graph (2) suggests that as at A and B the eggs may be isotonic with the two different media used, it is probable that C gives the osmotic concentration of the egg.

EXPERIMENT II.—*Effect of Hypotonic Media on the Development: Eggs*

(*a*) *On Eggs without Jelly.*—Numerous eggs removed from the jelly were exposed to different salinities. After every 24 hours of such exposure, 100 eggs from each lot were taken and the number of eggs that were dead* were counted. Column A in Table I gives the results of such a series of experiments. The figures indicate a rise in mortality correlated with a prolongation

TABLE I

Effect of Hypotonic Media on Development—On Eggs without jelly, with jelly and larvæ

Concentration of the medium ‰	A				B			C			
	Egg—Without Jelly				Egg—With Jelly			Larva			
	24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	72 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Distilled water	..	..	..	..	5%	7%	nil	..	..	..	..
11·40	..	..	..	..	3%	6%	nil	..	..	..	..
14·45	..	..	..	..	..	..	..	100%	100%	100%	100%
15·39	..	..	..	..	2%	5%	nil	..	..	..	..
15·65	..	..	..	..	..	..	..	53%	51%	53%	53%
16·82	..	..	..	..	..	..	..	24%	22%	22%	23%
17·50	..	..	..	..	..	..	..	2%	nil	nil	nil
17·78	33·3%	86·6%	88·3%	100%	..	..	..	..	..	..	..
17·86	..	..	..	..	6%	7%	nil	..	..	..	..
18·42	10·0%	83·3%	84·3%	100%	..	..	..	..	..	..	..
20·63	..	..	..	..	3%	3%	nil	..	..	..	..
20·65	..	..	..	..	..	..	..	2%	nil	nil	nil
21·88	..	..	..	..	..	..	..	2%	nil	nil	nil
22·18	..	..	..	..	..	..	..	1%	nil	nil	nil
24·33	..	..	..	..	4%	3%	nil	..	..	..	..
30·12	6·6%	66·6%	100·0%	100%	..	..	..	..	..	..	..
31·05	..	..	..	..	..	..	..	1%	nil	nil	nil
31·11	..	..	..	..	nil	nil	nil	..	..	..	..
31·30	..	..	..	..	..	..	..	nil	nil	nil	nil
33·42	..	..	..	..	..	..	..	nil	nil	nil	nil

of exposure, till all the eggs died after 96 hours. The table also indicates that the mortality increases in media of lower salinity.

(*b*) *On Eggs with Jelly.*—A single, complete and uninjured egg case was allowed to develop in each of the different media of known salinities. After

* Complete cessation of the setting movement of the yolk indicated the outset of death.

a duration of every 24 hours the number of eggs that were dead were counted. It is evident from Column B in Table (I) that the rate of mortality is comparatively low and at the end of 72 hours all the eggs hatched out into larvæ irrespective of the salinity of the outside media in which they were left.

B. *Experiments on Larvæ (Initial Metatrochophore)*

EXPERIMENT I.—*Hypotonic Media and Its Effect on Volume Changes*

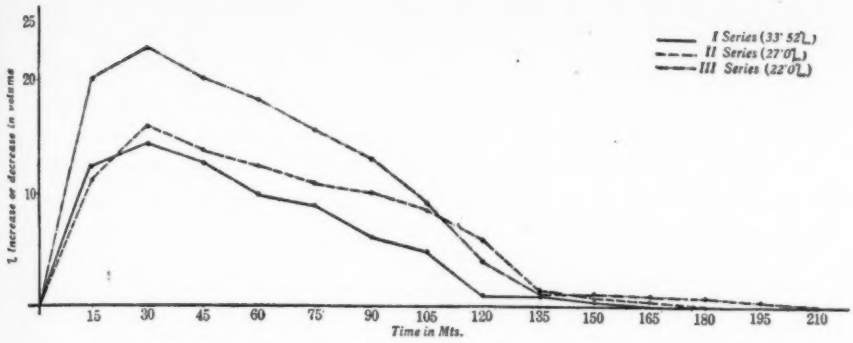
(*a*) *Increase in Volume in Different Hypotonic Media.*—The volumes of the larvæ hatched in the laboratory were first determined. Six of them were left in each of the seven petri dishes containing different concentrations of brackish water of the natural habitat of the worm. The volumes of these larvæ were measured after 30 minutes in each case so as to determine the effect of the different media on the volumes of the larvæ. Table II shows that in and beyond a salinity of 14·43 °/₀₀ the larvæ swell and disintegrate.

Concentration of the medium ‰	Laivae Initial mean volume 72C μ V	%
Distilled water	DISINTEGRATES	
7·43 ..	..	..
9·43 ..	DISINTEGRATES	
10·83 ..	..	..
13·31 ..	..	..
14·43 ..	DISINTEGRATES	
16·72 ..	..	..
17·76 ..	91·33	24·0
19·20 ..	..	..
21·10 ..	88·33	22·7
26·01 ..	..	..
26·10 ..	84·50	17·4
34·14 ..	81·66	13·4

(*b*) *Volume Changes in Hypotonic Media during Different Intervals.*—A number of larvæ were exposed to hypotonic media of different concentrations graded from 33·52 °/₀₀ to 22 °/₀₀ in order to determine their behaviour at different intervals. They were exposed for 3½ hours and the volume measured at an interval of every 15 minutes. Graph (3) shows that in all cases the original volume was regained after a duration of 3½ hours and that the larvæ are capable of reaching osmotic equilibrium in the different concentrations of the media.

EXPERIMENT II.—*Hypotonic Media and Their Effects on Development: Larvæ*

A number of larvæ of same age were allowed to continue their development in nine different grades of hypotonic media of known salinities in order to test their effect on the development of the larvæ. Controls were also



GRAPH 3. Volume Changes in Hypotonic Media during different intervals

maintained. After every 24 hours the rate of mortality was calculated and Column C in Table I shows the readings of such a series of experiments. The experiments were continued for a period of 96 hours at the end of which period all the larvæ developed into the next stage. The low rate of mortality bears testimony to the fact that the larvæ continued their development irrespective of the low salinity of the media in which they were allowed to develop. It is further evident that larvæ are not able to tolerate a medium which falls below a salinity of 14·45 $^{\circ}/_{\circ\circ}$.

DISCUSSION

The investigations of Loeb and Westeneys (1915) on the eggs of *Fundulus heteroclitus* are of interest since they found that the eggs of this marine fish can develop and hatch in distilled water. They conclude that the protoplasmic membrane of these eggs is impermeable to salts and almost impermeable to water. Ramult (1925) who studied the influence of salt solutions upon the development of *Daphnia* eggs, Gray (1920, 1932) and Krogh and Ussing (1937) who studied the eggs of *Salmo*, a fresh water trout, have come to a similar conclusion since they found a normal and healthy development in all of them due to the impermeable membrane, conserving and protecting the internal concentration. The envelopes of the eggs of the present form show that they are permeable both to water and salts and exposure for about 24 hours to a hypotonic medium of 17·78 $^{\circ}/_{\circ\circ}$ proves harmful for the normal development of about 33·3% of the eggs. If the exposure is for 48 hours the percentage of mortality of the eggs rises to 86·6%, until at the end of 96 hours all are dead. In a state of nature, however, the eggs are covered with jelly and changes in salinity through long periods of exposure do not affect the development of eggs. Therefore it can be concluded that the

envelop of jelly protects the eggs and serves the same purposes as the non-permeable egg coats of animals without jelly.

In the light of the observations made by August Krogh, Agnes Krogh and Wernstedt (1938) on the osmotic behaviour of the eggs of *Pleuronectes flesus* and *Crenilabrus exoletus*, teleosteon fish, the conclusion that the eggs of the present form are permeable both to salts and water seems to be justifiable. The behaviour of the eggs, which these authors studied, show that they decrease in diameter in water of 25–34 $^{\circ}/_{\circ\circ}$ at first, but increased to the original later. The eggs of the Polychæte, *Marphysa gravelyi*, also decreased in diameter in water of 29·73 $^{\circ}/_{\circ\circ}$ in the first 45 minutes but increased to the original in 3 hours, suggesting that they are permeable both to salts and water. Needham (1930), Ephrussi and Rapkine (1928) who studied the osmotic behaviour of the eggs of *Strongylocentrotus lividus* during their development stress the fact that they absorb large amounts of salts from the surrounding water. Ranzi (1930) observes that the eggs of *Sepia officinalis* increase in weight, ash content and water content during development. But in the present form shrinkage in volume due to loss of water and salts is seen. It is quite probable that due to the loss of salts and water which are quite essential for a normal and healthy development the eggs die and the rise in the percentage of mortality is directly proportional to the length of exposure. But the initial increase in volume in the eggs of the present form is probably due to the fact that the rate of inflow of water is greater than the rate of loss of salts, which indicates that the eggs must have had a greater concentration at the beginning. Similar conclusions were arrived at by Lillie (1916, 1918), Northrop (1926–27) and Runnstrom (1925) who observed an increase in the volumes of the eggs of echinoderms when they were subjected to the effect of different hypotonic media.

The experiments regarding the effect of different hypotonic media on the volume changes of the larvæ suggest that when they are exposed to different diluted media there is an inflow of water through the skin. But when they are exposed for a long time, they show a recovery of the original volume, indicating loss of water probably through the excretory organs. Since nephredia of the protonephridial type are already developed at this stage of the larvæ, it can be supposed that the excretory organs are responsible for osmo-regulation. Westblad (1922) who came to a similar conclusion, emphasises the fact that the flame cell system in the turbellaria is mainly concerned with osmo-regulation. Herfs (1922) by observing the rate of pulsation of flame cell system in rotifers and trematodes in solutions of different osmotic pressure, has obtained more direct evidence of their osmo-regulatory action. In the light of the above observations it is justifiable to

conclude that the recovery of the original volume, through the excretion of the water taken up during swelling, and the maintanance of a constant internal concentration, must be due to the activity of the protonephridial organs in the larvæ.

Krogh and Ussing (1937) further observe that the production of impermeable plasma membranes is probably a general mechanism for the protection of eggs in fresh water against osmotic swelling, a protection which can be dispensed with when mechanisms for the excretion of water become functional in the embryo. The observations of Ikeda (1937 *a*) on the eggs of *Oryzias latipes*, a fish living in fresh water and brackish waters of Japan, furnish another example of this kind. Since in the present form there is already a larval kidney (of the type of protonephredia) functioning, the larvæ seem to have dispensed with the protection afforded by the jelly. The almost insignificant low rate of mortality of the larvæ when they are exposed to different hypotonic media further strengthens the conclusion that the larvæ at this stage are able to maintain a steady internal concentration due to the presence of the functional kidneys which begin to actively excrete water.

The different modes of behaviour as exhibited by the eggs and larvæ when they are exposed to the effects of similar hypotonic media, further suggest that the eggs can scarcely be supposed to possess mechanisms specially adapted for invasion of water with fluctuating salinities, whereas the larvæ are endowed with well-developed excretory organs which serve as the osmoregulatory mechanism, as evidenced by the present investigations.

Summary

1. Experiments regarding the effects of the hypotonic and hypertonic media on the volume changes are described and the conclusion that the eggs are passive as far as the transport of the salts and water are concerned is arrived at.

2. The rate of mortality observed when eggs with jelly and without jelly were subjected to hypotonic media is recorded and the conclusion that the envelop of jelly acts in the same way as an impermeable membrane in the case of eggs of other animals is drawn.

3. The effects of hypotonic media on the volume of larvæ is described and the role played by the excretory organs and their importance in osmoregulation is indicated.

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STUDIES IN SAMPLING TECHNIQUE

V. Estimation of Borer Incidence in Sugarcane

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Introduction

Top, stem and root borers are among the major pests of sugarcane (Plates VIII and IX) and the activity of one or the other is noticed throughout the growing season of the crop. The estimation of the incidence of borers on sugarcane is needed as a prerequisite for the application of control measures as also for an appraisement of the damage done to the crop. The calculation of the incidence requires the splitting of canes causing as a result the loss of a considerable number of clumps. There is, therefore, an obvious necessity for a suitable sampling technique affording a dependable estimate of the incidence based upon the inspection of the minimum number of cane stalks in the field. Preliminary sampling studies on borer incidence have already been reported (Khanna, 1944), where it was indicated that the calculation of the incidence of borers based on the inspection of 15 clumps taken at random from a field of 1/40th acre would furnish a dependable estimate of the incidence. This procedure is being adopted in an appraisement of the damage done to the crop under field conditions during the survey of the diseases and pests in sugarcane in Bihar, which is an integral part of the co-ordinated scheme for crop surveys executed by the station from year to year. The sampling studies revealed further that the number of clumps in the ultimate sample size is an important consideration in increasing the precision of the incidence estimates, the efficiencies being of the same order with an equal number of clumps in the different sampling procedure. It is now proposed to furnish a detailed account of the variations of the incidence at different stages, bringing the sampling studies at the same level as with those already reported on whitefly (1948, 1950), pyrilla (1950) and mite (1950).

2. Material and the Basis of Recording Incidence

Four plots of 1/40th acre in size (72′ × 15′) were marked out at random from each of two fields (1·5 acres) given to B.O. 11, and Co 453, which were infested with borers. The rows were divided into lengths of 3′ units,

and the clumps and canes were serially counted in each. The details of the incidence due to borers have subsequently been recorded as per Form No. 1.

Usually borer incidence is recorded as a percentage on the number of affected canes and nodes, as also on the basis of the damaged length of the stalks. Each of these bases has its special significance in relation to incidence. The percentage infestation on the basis of length has a decided advantage in sampling studies, as it furnishes a fairly dependable index of both incidence and its intensity, which are associated concepts in such studies, and as such, the sampling studies in this note have been confined to the incidence on the basis of length alone. Table I given below shows the details of clumps, canes and the combined incidence due to all borers (length basis) in respect of all the 4 plots under the two varieties.

TABLE I

Showing the details of clumps, canes and the borer incidence in respect of the different plots

	Plot I	Plot II	Plot III	Plot IV
		B.O. 11		
1 No. of clumps ..	217	218	229	194
2 No. of canes ..	624	722	724	629
3 No. of clumps per unit ..	1·81	1·82	1·91	1·62
4 No. of canes per clump ..	2·88	3·31	3·16	3·24
5 Percentage of borer incidence* .. (Length Basis)	1·99	2·63	2·34	2·42
		B.O. 453		
1 No. of clumps ..	207	221	216	193
2 No. of canes ..	791	695	736	741
3 No. of clumps per unit ..	1·73	1·84	1·80	1·61
4 No. of canes per clump ..	3·82	3·14	3·41	3·84
5 Percentage of borer incidence .. (Length Basis)	1·54	1·57	1·40	1·62

* Without transformation.

The figures of percentage incidence will show that there has been a mild infestation in the fields. The information on the number of clumps per unit has its own necessity, as it prescribes the limit of the number of clumps to be included in the various hypothetical sampling combinations studied in this note in connection with the evolution of minimum sample size as against the desired level of accuracy.

Chart (1·1) *showing the incidence* (*Length basis*) *due to top borer*

(Co 453)

Rows \ Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
I ..	0·4 5	5·10	3·39	3·92	0·70	0·77	0·33	0	0	3·86	1·94	3·62	0·99	2·90	0	0	0	0	G	0	0	0	G	1·19
II ..	0·61	2·25	0·69	1·03	2·81	5·85	1·09	0·52	2·51	2·48	0·94	0·68	G	0·34	G	0	0	G	0·57	0	0·81	0·59	0	0·36
III ..	0	0·52	1·08	0	0	0	0·26	0	0	0	0·37	0	0	0	0·37	0	0	0	0	0	0·70	0	1·88	G
IV ..	0	1·69	1·35	1·95	0·81	2·56	0	1·32	1·21	0	0·78	0·47	0	1·08	0	0·80	0·98	1·09	0	0·68	0·70	0·53	2·11	2·26
V ..	1·62	0	0	0	0	1·69	1·44	1·24	1·59	0	0·85	0	G	0	0	2·96	0	0	0	0·19	0	0·77	0	0

Chart (1·2) *showing the incidence* (*Length basis*) *due to stem borer*

(Co 453)

Rows \ Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
I ..	0	0	0	0	0	0	0	0	0	0	0	1·85	1·90	2·68	0	0	0	0	G	0	0	0	G	0
II ..	1·37	0·86	1·39	1·03	0	0	0	0	0	0	0	0	G	0	G	0	0	G	0	0	0	0	0	0·63
III ..	0	0	0·16	0	2·23	13·0	0·26	0·69	1·12	0	0	0·81	2·41	0	0	5·86	0·68	2·12	0·60	0	3·49	1·15	0	G
IV ..	0	0	0	0	0·65	0	0·23	0·65	0·40	0·37	0	0·59	0	0·40	0	0·72	0·59	3·15	1·27	0·27	0	0·27	0	0·34
V ..	0	0	0	0·64	2·28	0	0	1·42	3·52	2·93	1·53	0	G	0·84	0	0	0·61	0	0	0	1·12	0	0	0

0 means no incidence in the unit.
G „ no cane (gap) in the unit.

Chart (1·3) *showing the incidence* (*Length basis*) *due to root borer*

(Co 453)

Rows \ Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
I ..	0·57	0	0·20	0	0	0·77	0·22	0·32	0·19	0·34	0	0·53	0·45	0·45	0·60	0	0·41	0·10	G	0	0	0	G	0
II ..	0·84	0·21	0·23	0·64	0·94	0·39	0·36	1·05	0·97	0·50	0·75	0·34	G	0·68	G	0·16	0·29	G	0·43	0·44	0·61	0·47	0·46	0·32
III ..	0·48	0·79	0·26	0·84	0·29	0	0·06	0·21	0	0·51	0	0·05	0	0·55	0·12	0	0·24	0	0	0·44	0·14	0	0·73	G
IV ..	0·31	0·67	0·50	0	0·54	0·43	0·57	0·92	0·20	0·25	0·53	0·23	0·31	0·13	0·47	0·46	0·39	0·98	0	0·14	0	0·13	0·07	0·34
V ..	1·22	0·50	0	1·41	0·17	0·24	0·48	0·97	0·23	0·42	0·34	0	G	0	0·06	2·22	0	0	0·40	0·19	0·19	0·39	0·28	0·49

Chart (1·4) *showing the combined incidence* (*Length basis*) *due to all borers*

(Co 453)

Rows \ Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
I ..	1·02	5·10	3·59	3·92	0·70	1·53	0·54	0·32	0·19	4·19	1·94	5·99	3·33	6·03	0·60	9	0·41	0·10	G	0	0	0	G	1·19
II ..	2·82	3·33	2·31	2·70	3·74	6·25	1·45	1·57	3·48	2·97	1·70	1·03	G	1·01	G	1·58	0·29	G	1·00	0·44	1·42	1·05	0·46	1·31
III ..	0·48	1·31	1·46	0·84	2·52	13·00	0·58	0·89	1·12	0·51	0·37	0·86	2·41	0·55	0·49	5·86	0·92	2·12	0·60	0·44	4·33	1·15	2·62	G
IV ..	0·31	1·76	1·85	1·95	2·07	2·99	0·80	2·89	1·81	0·61	1·36	1·29	0·31	1·61	0·47	1·93	1·95	5·21	1·27	1·09	0·70	0·94	2·18	2·94
V ..	2·85	0·50	0	2·17	2·45	1·93	1·92	3·63	5·34	3·35	2·71	0	G	0·84	0·06	5·17	0·61	0	0·40	0·38	1·31	1·16	0·28	0·49

0 means no incidence in the unit.
G „ no cane (gap) in the unit.

3. Combined Incidence

Charts (1–4) are shown as specimen of the type of the infestation due to the top, stem and root borers separately. The infestation shown in the charts refers to one of the plots under Co 453. It will be noticed that there has been no infestation in a considerable number of the units. For simplicity in statistical analysis, as also for other practical consideration, the incidences due to the different borers have been combined, the distribution of the combined incidence in the 3′ units being of the nature as shown in the last diagram of the Chart.

4. Procedure of the Statistical Analysis in the Variation Studies

The variations in incidence due to plots, rows, units and clumps may be estimated with the help of standard formula for fourfold multistage sampling (1940, 1944), the plots, rows, the units and the clumps being denoted as the zones (strata) of successive orders. The formulæ are reproduced below for easy reference.

Let p be the total number of variates, and p_i, p_j, and p_{ijk}, the number of the 4th order variates in the selected sample units of the 1st, 2nd and 3rd order zones (strata) respectively.

Let V_1, V_2, V_3 and V_4 represent the successive variances of the zones in the structure of the analysis of variance. Then V_4 will be the unbiassed estimate of 6_4^2

$(m-n)\,V_3$ will be the unbiassed estimate of

$$\left\{ p - \Sigma_i \Sigma_j \frac{\Sigma_k p_{ijk}^2}{p_{ij}} \right\} \sigma_3^3 + (m-n)\,\sigma_4^2$$

$(n-t)\,V_2$ will be the unbiassed estimate of

$$\left\{ p - \Sigma_i \frac{\Sigma_j p_{ij}^2}{p_{ij}} \right\} \sigma_2^2 + \left\{ \Sigma_i \Sigma_j \frac{\Sigma_k p_{ijk}^2}{p_i} \right\} - \Sigma_i \frac{\Sigma_j \Sigma_k p_{ijk}^2}{p_i} \sigma_3^2 + (n-t)\,\sigma_4^2$$

$(t-1)\,V_1$ will be the unbiassed estimate of

$$\left\{ p - \frac{\Sigma_i p_i^2}{p} \right\} \sigma_1^2 + \left\{ \Sigma_i \frac{\Sigma_j p_{ij}^2}{p_i} - \frac{\Sigma_j \Sigma_j p_{ij}^2}{p} \right\} \sigma_2^2$$

$$+ \left\{ \Sigma_i \frac{\Sigma_j \Sigma_k p_{ijk}^2}{p_i} - \frac{\Sigma_i \Sigma_j \Sigma_k p_{ijk}^2}{p} \right\} \sigma_3^2 + (t-1)\,\sigma_4^2$$

where σ_1^2, σ_2^2, σ_3^2 and σ_4^2 are the true variances due to the successive zones (strata) and t, n and m have the same significance as given below. We have

$$V(X\ldots.) = \frac{\Sigma_i p_i^2}{p^2} \sigma_1^2 + \frac{\Sigma_i \Sigma_j p_{ij}^2}{p^2} \sigma_2^2 + \frac{\Sigma_i \Sigma_j \Sigma_k p_{ijk}^2}{p^2} \sigma_3^2 + \frac{1}{p} \sigma_4^2.$$

In equal case (*i.e.*, where the number of variates is equal from sample unit to sample unit in the strata), the above formula reduces to

$$V(X....) = \frac{\sigma_1^2}{t} + \frac{\sigma_2^2}{n} + \frac{\sigma_3^2}{m} + \frac{\sigma_4^2}{p}$$

where t, n and m are the total number of variates in the successive zones (strata).

5. Variations in the Different Strata

Three rows out of five have been selected at random and in each row random selection has been made of 2 units giving in all 6 units, which work out as 5% of the available units. Selection of the 6 units per plot as above has been repeated 4 times for both the varieties and the distribution of the variations has been studied in all the 4 replications. The clumpwise incidence figures have been subjected to square-root transformation before analysis and the successive zone variances V_1, V_2, V_3 and V_4 obtained from the usual structure of the analysis variance. The estimates of σ_1^2, σ_2^2, σ_3^2 and σ_4^2 both in absolute values as well as in terms of percentages to the total variation have been shown in Table II for all the different replications. The errors as percentage of the mean incidence have varied from 9·14% to 13·19% in B.O. 11 and from 6·35% to 12·23% in Co 453. It is noticed that the variation due to clumps is always the maximum, varying from 60·07% to 79·33% in B.O. 11 and from 79·26% to 98·24% in Co 453. The variation due to plots has come out to be zero in all the 4 replications under Co 453 and in 2 replications out of 4 under B.O. 11.

In an attempt to see if it is possible to get still more steady estimates of the variances, 2 units per row (8·33% of the available units) were selected at random for inclusion in the samples. Variations have been studied only in 2 replications of this size. The estimated variances and the errors have also been set out in Table II. Even with this increased sample size, the errors have not been sufficiently low, thus pointing out towards the necessity of increasing still further the sample size in the estimation of the variances due to the different strata. The estimates of the variances have finally been estimated from the complete material. The errors have been 2·96% and 2·09% in B.O. 11 and Co 453 respectively. Hence it is obvious that it will be necessary to bring under inspection a considerable amount of the sampling material to bring down the error of estimation to less than 5% of the mean incidence. This is due to the fact that there is a considerable amount of inherent non-homogeneity in the incidence data.

TABLE II

Showing estimates of the variances and the errors as obtained under the samples and the whole plot

Samples	Mean	σ_m expressed as % of the mean	Zone Variances							
			Absolute value				Expressed as % of the total			
			B.O. 11							
			σ_1^2	σ_2^2	σ_3^2	σ_4^2	σ_1^2	σ_2^2	σ_3^2	σ_4^2
5% sample I ..	1·64±0·2163	13·19	0·1061	0·0000	0·1124	0·6322	12·47	0·00	13·21	74·32
do II ..	1·77±0·1726	9·75	0·0150	0·1351	0·1042	0·3825	2·36	21·22	16·36	60·07
do III ..	1·66±0·1860	11·20	0·0000	0·1926	0·0000	0·7394	0·00	20·67	0·00	79·33
do IV ..	1·62±0·1480	9·14	0·0000	0·0818	0·0723	0·5491	0·00	11·63	10·28	78·09
8·33% sample I ..	1·66±0·1140	6·87	0·0000	0·0000	0·0000	0·8427	0·00	0·00	0·00	100·00
do II ..	1·88±0·1513	8·05	0·0014	0·0404	0·2388	0·9797	0·11	3·21	18·94	77·74
Complete material ..	1·60±0·0474	2·96	0·0000	0·0278	0·0196	0·6713	0·00	3·87	2·73	93·40
			Co. 453							
5% sample I ..	1·47±0·1104	7·51	0·0000	0·0120	0·0000	0·4952	0·00	2·37	0·00	97·63
do II ..	1·44±0·1761	12·23	0·0000	0·1873	0·0000	0·6395	0·00	20·74	0·00	79·26
do III ..	1·33±0·1400	10·53	0·0000	0·0145	0·0000	0·8073	0·00	1·76	0·00	98·24
do IV ..	1·46±0·0927	6·35	0·0000	0·0000	0·0148	0·3576	0·00	0·00	3·97	96·03
8·33% sample I ..	1·37±0·0810	5·91	0·0000	0·0000	0·0000	0·4666	0·00	0·00	0·00	100·00
do II ..	1·44±0·1659	11·47	0·0548	0·0000	0·0000	0·9336	5·54	0·00	0·00	94·46
Complete material ..	1·36±0·0341	2·09	0·0000	0·0108	0·0000	0·5182	0·00	2·04	0·00	97·96

1. σ_1^2, σ_2^2, σ_3^2, and σ_4^2 represent the estimated variances due to successive zones.

2. In case the estimate of the variance due to a particular stratum (say V_3) is lower than that due to the next higher order stratum (*i.e.*, V_4), the estimate of the corresponding true variance (*i.e.*, σ_3^2) is shown as zero.

6. SAMPLING PROCEDURE

It is noticed that the relative magnitudes of the variances as given by the complete material are slightly different from those obtained under the two sample sizes. From a study of the distribution of errors as also from the steadiness of the estimates of the variances given by the complete material, it appears, it would be appropriate to use these estimates in the study of the probable sampling combinations.

Table III shows the hypothetical sample sizes in terms of the number of plots × number of rows × number of units × number of clumps, which would afford the estimation of the incidences within an error margin of 5%, 7% and 10% respectively.

Four plots were selected at random and the plot size was so chosen that there were only 5 rows in each. In the hypothetical combinations, the numbers of plots and rows have therefore been kept fixed at 4 and 5 respectively allowing variations only in the number of units. There is no justification

for going beyond one clump per unit in the hypothetical combinations, as the number of clumps per unit of 3′. length has been invariably less than 2.

TABLE III

Showing hypothetical combinations as against the different levels of accuracy in estimation

Error	Plot	Rows	Units	Clumps	Unit sampled		Clump sampled	
					No.	%	No.	%
			B.O. 11					
As 5% of the mean ..	4	5	6·9	1	138	28·75	138	16·08
As 7% of the mean ..	4	5	3·1	1	62	12·92	62	7·23
As 10% of the mean ..	4	5	1·4	1	28	5·83	28	3·26
			Co. 453					
As 5% of the mean ..	4	5	6·3	1	126	26·25	126	15·05
As 7% of the mean ..	4	5	3·0	1	60	12·50	60	7·17
As 10% of the mean ..	4	5	1·4	1	28	5·83	28	3·35

The table indicates what fractions of the number of clumps and units available in the complete material have to be included in the sample for an estimation of the incidence with an error margin of 5%, 7%, or 10%. It is interesting to notice that the requirement of the percentage clumps to be sampled has been almost exactly the same for both the varieties.

SUMMARY

Four plots of 1/40th acre in size (72′ × 15′) have been marked out at random from two sugarcane fields of 1·5 acres in area given each to B.O. 11 and Co 453, for sampling studies in the estimation of borer incidence. Each of the 5 rows in a plot has been divided into units of 3′ length and the incidence due to borers on the length basis has been recorded clumpwise for all the available clumps in the units. The complete material has subsequently been utilised in the estimation of the variations due to plots, rows, units and clumps with the help of the standard formulæ for fourfold multistage sampling. The variances due to the four hierarchies (strata) have been made use of in working out the probable sampling combinations of plots × rows × units × clumps (as shown below) such that the error of estimation may not exceed 5%, 7% and 10% of the mean incidence due to borers.

Error	Plots	Rows	Units	Clumps	Percentage of clumps sampled
	B.O. 11				
As 5% of the mean ..	4	5	6·9	1	16·08
7% of the mean ..	4	5	3·1	1	7·23
10% of the mean ..	4	5	1·4	1	3·26
	Co 453				
As 5% of the mean ..	4	5	6·3	1	15·08
7% of the mean ..	4	5	3·0	1	7·17
10% of the mean ..	4	5	1·4	1	3·35

The outermost column in the table given above shows what fractions of the available clumps have to be sampled in order to get the estimated incidence at the respective levels of accuracy—indicated in the first column.

Acknowledgements

The work was carried out as part of the Sugarcane Research Scheme in Bihar being financed jointly by the State Government of Bihar and the Indian Central Sugarcane Committee, to whom grateful thanks are due. Assistance rendered in the collection of the field material by Messrs. Md. Imran, S. N. Roy and R. P. Singh and in the statistical computation and analysis by Messrs. K. P. Singh and B. Acharya is acknowledged.

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A. Cane Stalk showing incidence of Top, Stem and Roof Borers

B. The Same Stalk Split open to show the incidence of and from the three Borers mentioned under 'A'

FORM No. 1

INCIDENCE OF BORERS

Variety.....

Date.....

Reference No.....

Sl. No.	Details of cane				Nodes and length bored and number of damage or insects present												Fungus diseases	Juice S. P.	Remarks
					TOP			STEM			ROOT			OTHERS					
	No.	G.	Wt.	Nd.	St.	Nd.	Lg.	St.	Nd.	Lg.	St.	Nd.	Lg.	Kind	Nd.	Lg.			
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

NATURE AND NURTURE IN YEASTS

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The Problem

The existence of multiple alleles at the locus which governs the nature of sculpturing of the giant colonies of our control two-chromosome brewery strain, BY 1 (Subramaniam, 1946) was surmised from observations on reverse mutations (Subramaniam, Ranganathan and Krishna Murthy, 1948). The classification of the various types in an ascending grade of complexity was shown to be a natural one (Subramaniam, 1950; 1951) as the simpler types appeared as orderly stages during the development of the complex ones.

Some peculiar types of sculpturing (Photos 1 and 2) were shown by some strains isolated after exposure to ultra-violet irradiation (Subramaniam and Krishna Murthy, 1948; Subramaniam and Ranganathan, 1949). All the previous conclusions were based on giant colonies grown under standardized conditions on Barley malt agar (Subramaniam and Ranganathan, 1948). The availability of a highly concentrated Ragi malt extract from a pharmaceutical firm suggested its use as a labour-saving device. Actually, it complicated issues. Some of the new strains isolated after ultra-violet irradiation were grown on Ragi malt agar. The problem arose as to whether the new types of sculpturing were the manifestation of new alleles or whether they were merely the altered expression of known alleles as a result of the use of Ragi malt agar. There are scattered statements in published literature (Skovsted, 1943; 1948) to show that alterations in the composition of the media affect the nature of sculpturing of the giant colonies. It became necessary under the circumstances, to clearly evaluate the effect of the composition of the medium on genic expression.

Material and Methods

Since mutations in different directions are sporadic, the effect of the environment on genic expression was investigated only at irregular intervals on the various mutant strains of our control. The photographs presented are the results of observations extending over a period of thirty months.

Colonies were grown at room temperature simultaneously on Barley and Ragi malt agar prepared from broths having a S.G. of 1·048 (Subramaniam, 1948; Subramaniam and Ranganathan, 1948).

Observations

There is no difference in the expression of the type, *Smooth I* in the two media. It was assumed by Subramaniam, Ranganathan and Krishna Murthy (1948) that the *Smooth I* was homozygous for the *Smooth* allele. When one of the *Smooth* gene mutates to the *Lace* allele the colony (*Smooth II*) shows pronounced concentric striations in its outer half (Photo 3). That is in Barley malt. In Ragi malt, on the other hand, the *Lace* gene brings about only an accentuation of the radial folding (Photo 4). This colony shows a sector having an accelerated rate of growth which appears to be governed by a separate but specific gene. Curiously enough the sector shows concentric striations. Only future investigations would show whether the gene determining growth rate has a modifying effect on those governing the nature of sculpturing of the giant colony. The divergence of the giant colony expression becomes more marked in the increasingly complex types. When a cell becomes homozygous for the *Lace* gene (*Smooth III*) the concentric and radial striations become prominent in Barley malt agar (Photo 5). In media prepared from Ragi, the radial folds show branching and there appear also minor folds in the outer half (Photo 6). No concentric striations are developed. The *Rough I* has been taken to have a genic constitution, *Smooth/Rough*. The only difference in the sculpturing between the *Smooth III* and *Rough I* (Photos 5 and 7) is the possession by the latter of a rough texture owing to the presence of minute granulations. In Ragi malt agar the branches of the radial folds become finer and closely spaced. No granulations are developed (Photo 8). All the above colonies were grown simultaneously in the two media under almost identical conditions. Unfortunately, the rarity of the *Rough II* condition did not enable such a direct correlation. Evidence, however, could be offered from other directions. A comparison of Photos 4, 6 and 8 of colonies grown on Ragi malt show a progressive accentuation of the radial folding. Giant colonies showing much more complex sculpturing (Photo 10) than that observed in Photo 8, appear to belong to the *Rough II* category (Photo 9). It has been shown previously (Subramaniam, Ranganathan and Krishna Murthy, 1948) that colonies exhibiting different types of sculpturing as sectors do appear periodically. Two such are illustrated in Photos 11 and 12. In Photo 11 there is a *Smooth* sector and as one proceeds in the clockwise direction, the *Smooth II* and *Smooth III* types could be distinguished. A colony showing sectors with

more complex sculpturing is illustrated in Photo 12. The small *Smooth* sectors can be classified as *Smooth II.* The rest are of two types. On the right-hand side, the complexly folded sectors do not show granulations. Hence they appear to belong to the *Rough I* class. The sector on the left-hand side—above the 9 O'clock position—shows in addition to the intricate folding, the presence of minute granulations. It should therefore be the *Rough II* variety. Photographs 11 and 12 offer proof that Ragi malt does not form a selective environment for any particular mutant.

Discussion

Yeast cells divide almost every hour in well aerated media. Even if the rate of mutation in yeast is identical with that in higher organisms, the shorter generation time results in a quicker appearance of the mutants in the cultures. This feature forms a stumbling block for any investigation of gene segregation by spore isolations. Accurate work would be possible only if (1) the mutational step is inhibited (Dickinson, 1945) by some agency or (2) specific types are cultured by offering them a selective environment (Krishna Murthy and Subramaniam, 1951). The mere fact that a single cell or single spore is isolated does not indicate any genetic purity as claimed by Winge (1951, p. 95). A relevant quotation from Skovsted may not be out of place. " Similarly in Nad. 2 single cells were isolated both from the normal one and from the sector. The results were rather confusing. Out of four cultures from the normal part two produced smooth giant colonies and two produced furfuraceous colonies (one with a small smooth sector). And of three cultures from the squamose sector two produced smooth colonies, whereas one consisted of both smooth and squamose parts " (1943, p. 413). Skovsted comments that " mutations had taken place while the cultures were being propagated in the nutrition solution before they were transferred to the gelatin " (1943, p. 413). When isolation of single spores or single cells does not ensure genetic purity, it is rather surprising that Winge (1951) comments that we have not worked with single cells or single spores. It has been our belief that unless methods are devised either to inhibit the mutational step or isolate a specific type by using a selective environment any attempt at conclusions based on spore segregation would only be of questionable value. It was with this idea that the factors governing gene expression were investigated. The fact that a specific type could be selectively grown (Krishna Murthy and Subramaniam, 1951) offers the hope that it would be possible to investigate gene segregation by spore isolations with much more reliability than has been done by Winge and his collaborators.

Because of the above difficulty, reverse mutations were employed to identify gene mutations (Subramaniam, Ranganathan and Krishna Murthy, 1948). This was in the belief that as stated by Muller (1939) reverse mutation " giving an apparently exact restitution, and of its fairly high frequency, thus furnishes an argument for our older view of the discreteness of the individual gene ". Using reverse mutations it has been possible to postulate the probable existence of multiple allelomorphs. It was emphasized that with the published evidence available those were the only rational interpretations possible (Subramaniam, Ranganathan and Krishna Murthy, 1948, p. 45).

Further investigations indicated that the simpler types of sculpturing appear as orderly stages during the development of complex ones. This justifies the belief that the alleles occurring at the locus appear to be related to one another as hypo- and hypermorphs (Subramaniam, 1950; 1951). Mallya and Subramaniam (1949) showed that different allelic combinations have different rates of growth at room temperature. This discovery was utilized to selectively isolate the *Rough II* by growing colonies in an incubator at 31° C. (Krishna Murthy and Subramaniam, 1951). Temperature has thus an entirely different effect on yeasts from that on *Drosophila* (Gordon and Sang, 1941; Stern, 1943). The effect of temperature on *Drosophila* is not comparable to that in yeasts for the simple reason that temperature " by changing the population of micro-organisms in the food, or the metabolic activity of these organisms " (Stern, 1943, p. 417) affect only indirectly the development of *Drosophila*. There is also another objection to any direct comparison of the results reported in *Drosophila* with those recorded in the present paper. The expression of a mutant gene in *Drosophila* though depending on the culture medium becomes really an ecological problem (Gordon and Sang, 1941, p. 181).

A perusal of the Photographs 3, 5, 7 and 9 would indicate the ascending grade of complexity exhibited by the different allelic combinations. It has been shown earlier (Subramaniam, 1950; 1951) that this classification of the various types is a natural one, since with increasing complexity of the final expression, the simpler types are not only pushed farther and farther back during development, but there is also a corresponding shortening of the duration of each stage. A comparison of Photos 4, 6, 8 and 10 with those of the standard types in Barley malt agar would suggest that the effects of the alleles are similar but find expression in different directions. This is what should be anticipated if the different alleles are related to one another as hypo- and hypermorphs.

It is quite likely that Barley and Ragi malts differ in their composition as regards assimilable sources of carbon and nitrogen and of the vitaminic constituents. Whether particular nutritional factors have any effect on genic expression is being systematically investigated.

Summary

1. The nature of sculpturing of the giant colonies of the control two chromosome brewery strain is different in Ragi and Barley malt agar.

2. The effects of the alleles are similar but find expression in different directions.

3. Photographs of comparable equivalents in the two media are presented.

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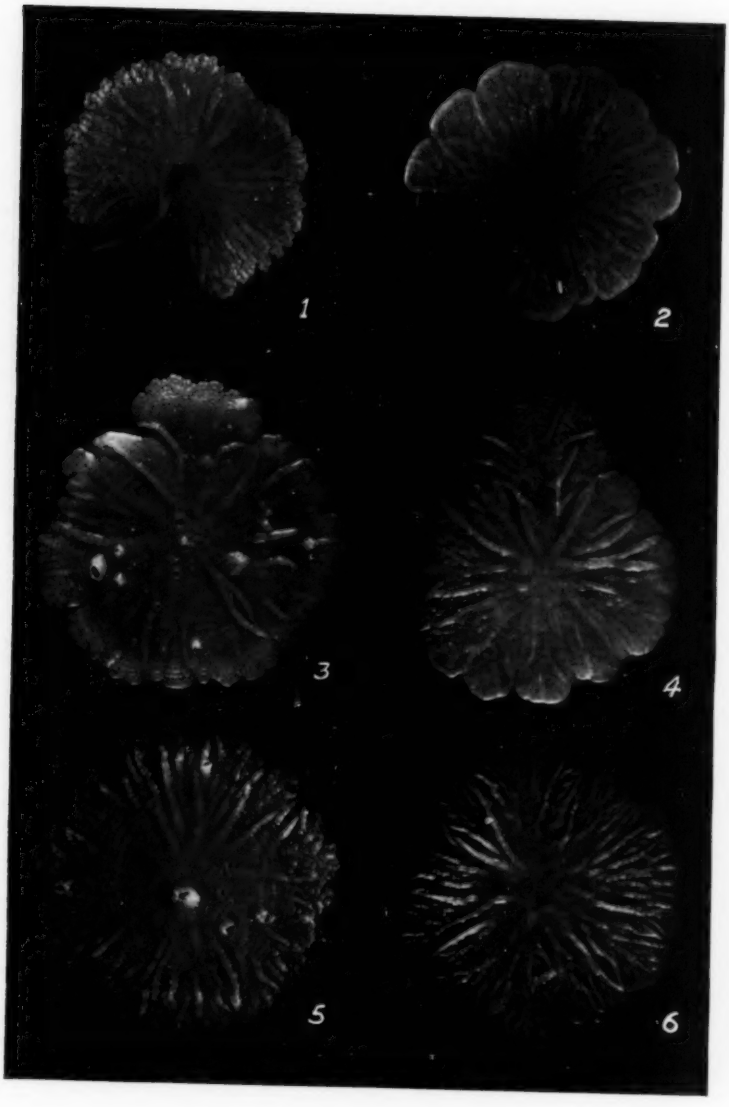
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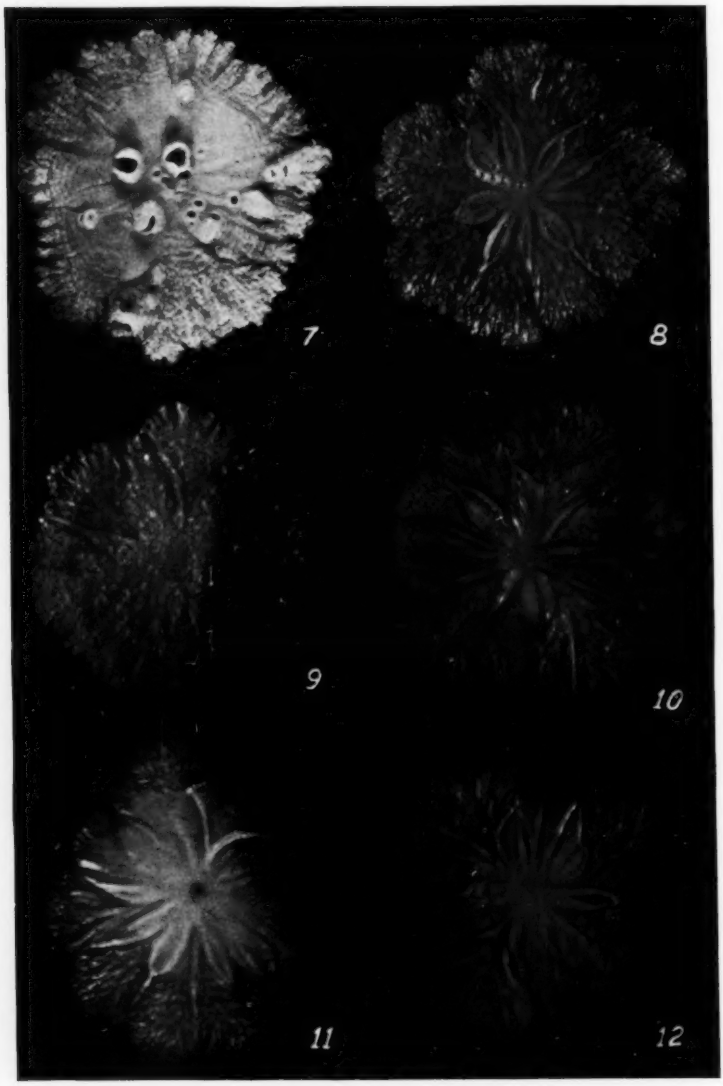
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Description of Photographs

Photo 1. BYU 4 (From BY 1 exposed to u-v irradiation). Ragi malt agar. 3·3 cm. 14-day growth. 10–10–47.

Photo 2. BYU 2 (From BY 1 exposed to u-v irradiation). Ragi malt agar. 3·7 cm. 14-day growth. 10–10–47. (After Subramaniam and Ranganathan, 1949).

Smooth II

Photo 3. BY 1 (19–12–45). Barley malt agar. 3·3 cm. 18-day growth. 16–8–48.

Photo 4. BY 1 (19–12–45). Ragi malt agar. 3·0 cm. 18-day growth. 16–8–48.

Smooth III

Photo 5. BYU 2 S. (From BY 1 exposed to u-v irradiation). Barley malt agar. 3·6 cm. 12-day growth. 24–8–48.

Photo 6. BYU 2 S. Ragi malt agar. 3·4 cm. 12-day growth. 24–8–48.

Rough I

Photo 7. BY 1 (12–10–45). Barley malt agar. 4·0 cm. 19-day growth. 26–10–48.

Photo 8. BY 1 (12–10–45). Ragi malt agar. 3·6 cm. 28-day growth. 26–10–48.

Rough II

Photo 9. BY 1 (12–10–45). Barley malt agar. 3·4 cm. 15-day growth. 22–10–48.

Photo 10. BY 1 (19–12–45). Ragi malt agar. 3·3 cm. 14-day growth. 29–4–48.

Mixed Types

Photo 11. BY 1 (12–10–45). Ragi malt agar. 3·3 cm. 23-day growth. 15–9–48.

Photo 12. BY 1 (12–10–45). Ragi malt agar. 3·4 cm. 18-day growth. 16–8–48.

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GENETICS IN RELATION TO PLANT BREEDING*

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As is well known the relations between genetics and plant breeding are so intimate and comprehensive that it is impossible to cover this field in a single lecture. I shall try, however, to summarize some of the essential points, and I shall also mention some examples of present-day attempts to utilize advances in theoretical cytogenetics for plant breeding purposes. Several of these examples represent data acquired by Swedish workers. I must apologize for this one-sidedness, but naturally it is easiest for me to speak of material with which I am more or less familiar.

Very soon after the rediscovery of Mendel's laws in 1900 it was realized that the new principles of heredity and variation had far-reaching consequences for practical plant breeding work. Already in 1901 and 1902 de Vries and Tschermak visited the Svalöf plant breeding station in Sweden and told the staff members about the new discoveries. In the first place the principle of genetic recombination must have appealed to the breeders, as it clearly demonstrated the possibility of combining in one variety various valuable characters originally present in different parent strains. Genetic recombination is still one of the two cornerstones of modern plant breeding. The other one, which is even more fundamental, is the demonstration by Mendel of stable hereditary units, later on called genes.

On this basis of stable genes which may be recombined, some other conceptions of primary importance were soon established. Thus, already in 1902, Bateson coined the terms homo- and heterozygote, and soon afterwards the Dane Johannsen made the distinction between genotype and phenotype. He also clarified the difference between genetically heterogeneous populations, containing individuals with different hereditary constitutions, and homozygous pure lines, in which all the individuals have the same hereditary constitution. He also demonstrated that selection was only effective as long as the material selected comprised different hereditary types. Selection within a really pure line gave only negative results, even if it was

* Based on a Lecture given at the Hyderabad Meeting of the Academy.

carried on for many generations, and this result was later on verified by other workers using various kinds of material.

The constancy of the genes also explained the constancy of clones, in which the same individual and the same genotype is reduplicated by various types of vegetative propagation. Ability of clonal propagation is extremely valuable in plant breeding work and has greatly facilitated the production of new improved varieties in such cultivated plants as potato, sugarcane and banana. Also in cocoa important results have recently been obtained by a change from seed reproduction to vegetative propagation.

Returning to material reproduced by seeds the possibility of selecting valuable new varieties by raising offspring from single individuals had already been empirically realized before Mendel and was especially practised by Louis de Vilmorin in the middle of the nineteenth century. Important results with this method in wheat, oats and barley were later on also obtained by Hays in Minnesota and by Hjalmar Nilsson and his collaborators at Svalöf. Otherwise the Darwinian views predominated among plant breeders in the nineteenth century, and the rather primitive procedure called mass selection was considered to be the best plant breeding method. This is very well illustrated by the following statement by the German plant breeder von Rümker: "All our cultivated plants constitute a plastic, malleable material which requires only the formative hand of the artist to become modelled into a thousand new racial forms, and just as not everyone can be an artist unless he has the necessary gifts and inspiration, so it is not everyone who is born to be a breeder."

Especially after the work of Johannsen it was realized that the isolation of new varieties by line selection could be carried out with success only in populations of self-fertilizing species, and even in such cases the method had its obvious limitations. It was found that by such selection nothing really new was produced and that only an isolation of hereditary types which were already present in the population took place. And further, in many cases, the constellation of characters shown by the extracted lines was far from ideal. If a new line represented an improvement in one respect it very often had taken a step backwards in another respect.

In a now classical paper Nilsson-Ehle (1906) points out the great difficulties of obtaining really good products by line selection in spontaneous populations and recommends artificial crosses as the best way of combining various desirable characters. He especially emphasizes that the crosses should be undertaken with the definite view of combining one or the other valuable property of one variety with certain other properties of the other

parent. Crosses should not be undertaken just in order to increase variation. Nilsson-Ehle gives a number of examples and says that in the offspring of artificial hybrids he had been able to get some derivatives combining valuable characters of the parents but also segregation products combining the poor properties of the parents. Thus, from the cross between the winter wheat varieties Extra Squarehead and Grenadier, some forms combined the short stiff straw of Grenadier with the resistance to yellow rust characteristic of Extra Squarehead, but other derivatives combined the tall and rather lax straw of Extra Squarehead with the susceptibility to yellow rust of Grenadier. Already from such examples it became clear that the economically important characters in wheat could be recombined.

In three papers published in the years 1908–11 Nilsson-Ehle clearly demonstrated that quantitative characters in general are inherited in a Mendelian way but that as a rule many so-called polymeric or multiple factors are involved in such cases. This theory was supported by comprehensive experimental data. Especially significant was the demonstration that after crossing and recombination it is possible to obtain numerous constant gradations with regard to different quantitative characters. This quantitative differentiation is evident already in F_1 but complete constancy is not obtained until later generations.

If the parents were widely different with regard to a certain quantitative character the constant gradations were predominantly intermediate between the parents. If, on the contrary, the parents were alike or almost alike, the gradations generally represented transgressions surpassing the limits of the parents. The frequent occurrence of positive as well as negative transgressions represents a very strong support of the theory of polymerism. The only possible interpretation is that quantitative characters in such cases are conditioned by several or even numerous constant genes which are recombined and that the parents carry different sets of such genes. The frequent occurrence of transgressions proved to be very valuable for breeding purposes, and Nilsson-Ehle considered the utilization of transgressions as a special breeding method.

An important consequence of the theory of polymerism, observed by Nilsson-Ehle, is that cross-fertilizing organisms are always heterozygous and represent an inexhaustible multitude of different genetic combinations. From the polymerism it became clear that it is not only the relatively few genes, having obvious visible effects which are segregating, but rather a large number of genes having small visible effects or only physiological consequences. Such segregation is still more masked by the modifying effects

of environmental influences. Under such circumstances quite special methods, vegetative propagation leading to clones or inbreeding, are needed in order to reveal the enormous hereditary polymorphism occurring within such an apparently homogeneous material as for instance the plants in a field of maize or rye.

When Nilsson-Ehle had seen that the polymeric genes generally conditioned such quantitative and physiological properties as must be of the greatest importance to the viability of the organisms and to their ability of surviving under variable environmental conditions, he concluded (in 1909) that the main purpose of sexual reproduction must be the creation of genetic recombination. This is a thesis which is now rather axiomatic, but not until the theory of polymerism had been developed was such a conclusion fully justified.

There is an immediate connection between the importance of genetic recombination to the viability of organisms and their adaptability to different types of environment. In one of his papers Nilsson-Ehle points out that not in a single case had he been able to find transitions from environmental modifications to heritable variations. Thus a Lamarckian explanation of the acclimatization of wild as well as cultivated plants was quite excluded. The mechanism at work was rather crossing, recombination and selection, this process also being promoted by spontaneous mutation. This represents the fundamental ideas of the special branch of science later on called genecology by Turesson and others. Nilsson-Ehle also gives numerous examples of the hereditary adaptation of cultivated plants to special habitats and shows that this is of great importance in plant breeding work. On account of this principle the central breeding station at Svalöf was later on supplemented by a network of branch stations in various parts of Sweden. As far as possible local selection and breeding is carried out at these stations. Only in this way it is possible to get a satisfactory hereditary adaptation to the ecological conditions characteristic of each local region.

It is well-known that the classical methods of plant breeding—selection in spontaneous populations as well as selection in populations produced by hybridization—have led to numerous improved varieties of great economical importance. This is especially true of the well-planned breeding by recombination, in which various desirable characters of the parents have been combined more or less successfully. As an example, I only need to mention the successful combination of the high specific yield of the English Squarehead wheat with the winter hardiness and good kernel quality of certain Swedish land varieties. The economic importance of such work may be illustrated by Table I.

TABLE I

The increase in value, due to plant breeding, of the cereal harvests in Sweden

Cereal	Present value of the total harvest (in kronor)	Per cent. increase due to plant breeding	Value of the increase (in kronor)
Winter wheat ..	99,863,750	25	24,965,937
Summer wheat ..	45,140,500	12	5,416,860
Winter rye ..	67,650,000	15	10,147,500
Oats ..	149,154,400	14	20,881,616
Barley ..	35,841,600	12	4,300,992
Mangkorn (oats+barley)	98,062,200	13	12,748,086

The table shows the value of the total yearly cereal harvest in Sweden, these values representing average values of the latest five-year period. The next column shows the increase in production which may be ascribed to plant breeding. These percentage values have been obtained by comparisons during several years between the old unimproved land varieties and the modern products of plant breeding grown side by side. The increases range from 12 per cent. in oats and summer wheat to 25 per cent. in winter wheat. The value of this increase may be seen in the last column. The total for all the cereals amounts to about 78 million kronor. This is the yearly increase in value which the Swedish harvest of cereals has attained thanks to plant breeding. It should also be kept in mind that the breeding has also resulted in other favourable changes which are not reflected by these values. This is, for example, true of the improvements in straw stiffness allowing a stronger nitrogen manuring. Improved kernel quality and an increased earliness are other advantages.

In Sweden much breeding work has also been done with other agricultural plants. It is not possible to make exact statements about these other crops, but it seems safe to conclude that plant breeding has been able to increase the annual value of the total Swedish crop by about 100 million kronor which corresponds to about 20 million dollars. This is a large amount for a small country like Sweden, especially if it is compared to the total amounts so far spent on plant breeding. For the period 1886 to 1948 these costs amount to about 15 million kronor and thus, the yearly profit is several times larger than the costs for the whole period of more than 60 years. The figures mentioned are of course far from exact, but, at any rate,

it is perfectly clear that the State of Sweden has made a brilliant bargain by its investments in plant breeding. Similar experiences have also been made in other countries, certainly also in India, and I do not think it is possible to find another business giving as good dividends as this one.

In cross-pollinating organisms it has been more difficult than in the self-fertilizing ones to attain definite knowledge of the hereditary constitution, and the results obtained in inbreeding experiments have been interpreted in various ways. However, most or all investigators agree that the differentiation occurring in inbred material is caused by homozygosity for genes present in the original, heterozygous population, and further, that hybrid vigour is due to hereditary differences between the parent types. As is well-known, hybrid vigour is especially striking after crosses between inbred lines.

It is very doubtful if the cytoplasm has anything direct to do with inbreeding degeneration as claimed by some workers. A rather strong argument against this possibility is represented by results recently obtained by one of my students, Mr. Lundqvist. He compares the effects of inbreeding in ordinary rye and in autotetraploid rye, having twice as many chromosomes, and finds significant differences, the tetraploids being much more resistant to inbreeding than the diploids. This may, indeed, be expected as quadrivalents are frequent at meiosis in the tetraploid and the segregation of homozygous recessives will be slow. In other words, the tetraploid rye has another chromosome mechanism than ordinary rye, and the results of inbreeding are in accordance with this difference in chromosome constitution. This shows that it must be the genes in the chromosomes which are exclusively or mainly responsible for the inbreeding degeneration.

The space available does not permit me to go into details concerning the different theories of inbreeding degeneration. I just want to call your attention to the extremely important population studies in Drosophila carried out by Dobzhansky and others which demonstrate a very frequent occurrence of recessive genetic factors which in a homozygous condition have more or less bad effects. They cause all degrees of reduced vigour from complete lethality to almost normal viability, and there is also preliminary evidence of factors decreasing fertility. Evidently this is all that is needed to explain a typical inbreeding degeneration. The occurrence of such factors in cross-fertilizing populations has long been assumed, but now their existence has been definitely demonstrated. The evidence is less definite in other organisms, but there is reason to believe that the situation found in Drosophila is also characteristic of most other cross-fertilizing species.

The practical utilization of all the knowledge gained from experiments on inbreeding and outbreeding is illustrated in the most beautiful way by the magnificent work on corn breeding carried out especially in the United States. The theoretical foundations in this field were created by such pioneers as Shull and East, and by a brilliant co-operation between many workers it has been possible to build up, step by step, a line material which on outcrossing has led to a very marked increase in yield. According to Sprague 83 per cent. of the maize grown in the corn belt of United States in 1944 was hybrid maize, and the use of this material led to an increase in yield of 600 million bushels.

Even if the details of the inbreeding phenomena are not yet quite clear I think we are fully justified in ascribing the practical results obtained to an intimate and unusually successful co-operation between theoretical genetics and practical plant breeding.

The development of genetics and cytology during the last decades has given rise to new plant breeding methods. This is especially true of the work on induced mutations and on polyploidy. After the discovery by Muller that mutations, permanent hereditary changes, may be induced by irradiation, and many other workers had concentrated on this new field of genetic research, attempts were also made to utilize induced mutations for plant breeding purposes. A special difficulty for these attempts is the fact that the induced mutants generally have a decreased viability, this reduction in vigour in the first place being caused by deleterious changes of the chromosome structure. But also many of the finer changes, which are considered to be true gene mutations, have a negative effect and are useless from a practical point of view. There remains, however, a quite small but exceedingly important group of induced mutations which are not destructive but in which viability is normal or even improved. It may also happen that mutations, having bad effects in their original genetical environment, may get a more favourable action after recombination. It is now perfectly clear that the new genes or alleles induced by irradiation are of essentially the same kind as the spontaneous ones, and this really means that the spontaneous rate of hereditary variation may be enormously increased by irradiation or other similar agencies..

Especially through the mutation experiments carried out by Gustafsson at Svalöf it is now perfectly clear that this method is of positive value to plant breeding. Especially in barley, the main material for this work, it has been possible to induce very marked and favourable changes in straw stiffness and earliness (Figs. 1–2). Some of the mutants also give a higher yield than the original material. This is shown in Table II taken from a recent publica-

TABLE II

Agronomic characters of thirteen induced mutants in barley

(Gustafsson and Mac Key, 1948)

Strain	No. of years tested	Rel. yield	Strength of straw (1–10)	1,000-grain weight, g.	Ripening time; days
Golden barley ..	8	100	6·5	38·1	103
Erectoides 1; 44/1 ..	8	102·2	7·3	37·0	104
Late, tall; 44/2 ..	8	103·3	8·0	38·4	107
Broad-leaved, late; 44/9 ..	5	100·5	7·6	36·2	109
Large-seeded; 44/7 ..	5	109·1	6·8	42·2	103
Broad-leaved 44/23 ..	6	102·0	6·6	41·5	105
Maja barley ..	4	100	8·3	39·0	105
Erectoides 12; 44/17 ..	4	101·9	9·1	39·0	106
Erectoides 13; 44/19 ..	4	96·1	10·0	36·5	105
Erectoides 16; 44/20 ..	3	98·1	9·3	40·1	99
Bright-green 2; 44/35 ..	4	99·8	9·0	38·8	106
Stiff, strawed, early; 44/18 ..	4	102·0	8·8	40·1	103
Seeds differently coloured; 44/18 ..	4	103·4	9·0	39·1	106
Broad-leaved, late; 44/31 ..	4	101·4	8·7	36·8	106
01513 b_7 ("Ymer") ..	3	100	9·8	42·1	103
Bright-green 3; 46/65 ..	3	102·0	9·3	41·0	101

tion by Gustafsson and Mac Key (1948). The values in this table have been obtained from field trials, carried out in the same way as in the testing of ordinary breeding material. Three commercial varieties are represented in the table together with thirteen selected induced mutations. The five mutations of Golden barley have been compared with the mother line for 5–8 years and all have higher values for yield as well as for straw stiffness. Some of them have an increased 1000-grain weight and this is especially true of the mutant "large-seeded; 44/7". On an average this mutant had 9·1 per cent. higher yield than the mother strain and yet it has the same earliness. The values of the other mutants in the table are somewhat less reliable, being based on the data from only 3–4 years. On an average, however, these mutants have good values and surpass the mother types either in yield, straw stiffness or earliness.

In practice Golden barley has now been replaced by the higher yielding varieties Maja and Ymer, and therefore the improved mutants of Golden barley are not able to compete with the new commercial varieties Maja and Ymer. Hence, the mutation work must now be concentrated on the varieties at present giving the highest yields. As is evident from the lower part of the table some promising mutants of Maja and Ymer have already been obtained. On the whole, I think the greatest value of the new mutants will

be as material for further breeding by recombination. Even if an extremely early mutant will have a lower yield than the mother line it is probable that the new mutant gene will be of value after recombination.

In several other plant species besides barley positive results of the same kind have been obtained by Gustafsson and his collaborators. This is true of wheat and oats as well as of yellow lupins and some other species. Efforts are now also being made to induce mutations in fruit trees.

TABLE III

Relative yields of the best barley mutants in Halle-Hohenthurm, 1947 and 1948

(Data given by Kuckuck-Mudra, 1950, p. 164)

No.	Mutant	1947	1948	1947–48 average
90	früher, kurzhalmig	123·30	114·6	118·95
96	früher, breite Bl. kürzer	127·89	101·4	114·60
120	früher	113·63	104·1	108·87
92	früher breite Bl.	110·06	105·6	107·83
115	standfester, kürzer, dunkler	104·48	104·0	104·24
3	erectum dunkel	104·20	101·7	102·95
245	dichte Ähre, gr. Korn, dunkel	101·47	102·5	101·99

Very promising results with induced mutations in barley have also been obtained by Freisleben, Lein and Hoffmann, working in Halle in Germany. From their very large material the following data are especially interesting (Table III). Though the period of testing is too short to allow safe conclusions these values strongly support Gustaffsson's conclusion that it is possible in barley as well as in other cultivated plants to get induced mutants of agricultural importance.

For many years and especially after the introduction of the well-known colchicine method of doubling the chromosome number attempts have been made all over the world to utilize induced polyploids in agriculture. At the Svalöf Institute a special department for such work was started in 1931. During its twenty years of activity plenty of material has been studied by my colleague Dr. Levan and by myself, and our experience may be summarized as follows:

1. Most induced polyploids have no practical value, but there is a small group of material in which the reaction to chromosome doubling is favour-

able. In such cases new material has been obtained which is already of great importance to agriculture or which most probably will be in the near future.

2. Only in rare cases are the primary polyploids, the so-called raw-polyploids, practically useful. In most cases it is necessary to improve this material by recombination and selection, and by such work vigour as well as fertility may be improved. The situation in this respect is about the same as with the induced mutations. Induction of polyploidy as well as of mutations implies a more or less violent disturbance of the hereditary balance, which may be partially overcome by recombination without losing the essential and practically important effect of the primary change.

3. Among the polyploids auto- as well as allopolyploids may be of practical value.

In support of these statements I wish to mention the following examples:

Diploid cross-fertilizing species in which the vegetative parts represent the agricultural products are an especially favourable starting material for the induction of autopolyploidy. Table IV represents a summary of field trials with diploid and tetraploid red clover (*Trifolium pratense*) which were carried out by Turesson, Levan and Frandsen. The values in the table are relative values concerning the green matter production, the diploids in each trial having the value 100. Thus values in the table higher than 100 imply that the tetraploid has been superior to the corresponding diploid. Considering the total harvest we find that in 11 cases out of 15 the tetraploids were superior to the diploids. The superiority is more marked at the second than at the first harvest per year. This means that the tetraploids are somewhat more slow-growing, but in red clover this is no disadvantage. A greater difficulty is the seed production which is lower in the tetraploids than in the diploids. However, according to Levan, selection for higher seed production is successful. In the related clover species *Trifolium hybridum*, the seed setting in the tetraploids is already sufficient. As also in this species the green matter production in the tetraploids is higher than in the diploids, two different tetraploid strains are already available for practical use. One of them was produced by Turesson, the other one by Levan.

Relatively favourable experiences with induced polyploids have also been obtained in beets. In sugar beets the triploids evidently represent the yield optimum, whereas the tetraploids are somewhat less good than the diploids. In turnips (Fig. 3) Levan has obtained quite good yield values for the tetraploids.

TABLE IV

Relative yield values in different strains of tetraploid red clover

(Data given by Levan, 1948, p. 488)

Strain	Year	Place of trial	Harvest			
			1	2	3	Total
ϕtofte ..	1945	ϕtoftegaard	101	116	131	112 } 114
tidlig ..	1946	(Frandsen)	109	147	..	119 }
	1944	Ultuna (Turesson)	..	125	..	..
		Svalöf	112	127	..	114
Merkur ..	1945	Ultuna (Turesson)	105	118	..	109
	1946	Svalöf	77	103	..	88
		Östergötland	115	132	..	120
		Västergötland	114	135	..	120
Heimsnap ..	1944	Ultuna (Turesson)	149	148	..	149
	1945	,, ,,	128	120	..	125
		Svalöf	82	98	..	87
Ultuna ..	1943	,,	77	119	..	90
	1946	Ultuna	106	57	..	96
	1943	Svalöf	104	113	..	107
Offer ..	1946	,,	102	113	..	105
		Västernorrland	103	..	..	103

From my own experience I want to give some information on tetraploid winter rye. Although this is an autotetraploid with ± 28 chromosomes instead of 14, it is quite vigorous and has remarkably good properties (Fig. 4). The degree of seed setting is somewhat reduced, it is true, and also the degree of tillering is less good in the tetraploids than in the diploids. However, as the kernel weight is more than 50 per cent. larger than in the diploids (Fig. 5), the total yield is rather satisfactory. Further it should be mentioned that the big kernels of the tetraploid give very vigorous seedlings and that the flour from these kernels has been found to have a better baking quality than the flour of ordinary diploid rye. Concerning resistance to frost and drought, earliness and straw-stiffness the tetraploids seem to be just as good as the corresponding diploids.

A difficulty for the evaluation of the tetraploids is represented by the fact that diploid and tetraploid strains cannot be directly compared in the same field trial. If tetraploid rye is exposed to the pollen of ordinary rye the degree of seed setting may be severely reduced owing to the formation of aborting triploid embryos.

It is therefore necessary to keep the diploid and tetraploid rye strains isolated from each other and to make indirect comparisons by the use of

common standards. I have used different varieties of winter wheat as standards, and trials of this kind have now been undertaken in five years at seven different places in southern Sweden. The values of one such trial are shown in Table V. In comparison to the wheat standard, Ergo, two diploid

TABLE V

Yield trial with diploid and tetraploid rye

(Skara, 1948)

Variety	Yield (water content 18%)		1000-grain weight
	kg/har	relative values	
Steelrye, dipl. ..	4260	130,3	30,3
Wasa II, dipl. ..	4190	128,1	29,3
Ergo wheat ..	3270	100,0	41,9
Steelrye, tetrapl. ..	5820	147,3	46,2
Wasa II, „ ..	5720	144,8	46,4
Crossing-group „ ..	5610	142,0	46,3
Ergo-wheat ..	3950	100,0	44,7

rye varieties, Steel and Wasa II, had the relative values 128 and 130 (Ergo = 100). In the tetraploid field trial the corresponding relative values of tetraploid Steel and Wasa II were 145 and 147 and thus clearly higher. The other values in the table demonstrate that in this particular case the 1000-grain weight was 55 per cent. higher in the tetraploids than in the diploids. In other cases the values of the tetraploids were not quite as good, and more data are needed before we can be quite sure about the relative yield of the diploid and tetraploid rye strains. However, the data already obtained justify the conclusion that yield in tetraploid Steel rye is not lower than in diploid Steel rye. As the baking quality of the tetraploid is decidedly superior it has now been decided to release tetraploid Steel rye to the Swedish farmers in this year. Large propagations of this variety were sown in the autumn of 1950.

My personal experience of induced allopolyploids in agricultural plants is limited to ryewheat, Triticale (Fig. 6) having 56 chromosomes, 42 wheat+14 rye chromosomes. In this connection I just want to mention that the best types available 15 years algo had only about 50 per cent. of the yield of the wheat standards. I have now Triticale-types reaching about 90 per cent.

of the yield of the wheat standard, and the possibilities of raising new and improved Triticale-strains are almost unlimited. I therefore believe that it will be possible to get Triticale-types of agronomic importance later on. The main practical idea behind the Triticale work is to get a new cereal giving better bread than rye, which could replace rye in sandy soil areas, in which wheat cannot be cultivated. Some Triticale strains have excellent baking properties, winter-hardiness and earliness and other strains a very good straw stiffness. Thus, the practically important properties are now distributed over the Triticale sortiment. To a certain extent, at least, it should be possible to combine them in one strain.

I can also mention that we have recently found it possible to combine wheat and *tetraploid* rye, though we do not know anything yet about the properties of this new allopolyploid.

Finally, I would like to say a word about the natural and synthetic allopolyploids in the genus Brassica. One of my students, Mr. Gösta Olsson, is working with various auto- and allopolyploids in this genus and especially with synthetic forms of winter rape (*Brassica napus*, var. Oleifera). This species has the somatic chromosome number 38 and represents a synthesis of winter turnip rape (*Brassica rapa* var. Oleifera) with 20 chromosomes and cabbage (*Brassica oleracea*) having 18 chromosomes. The following picture (Fig. 7) shows the appearance of a synthetic type of winter rape as well as the parents of the latter one. Synthetic rape has been produced by various workers, and Rudorf in Germany has already reported that hybrid derivatives between natural and synthetic rape forms have given quite promising results. In some cases the yield of these hybrids was 25–30 per cent higher than in the standard, a well-known commercial variety. Interesting and promising results with induced polyploidy in Brassica have also been obtained by a group of Indian scientists, working at the Indian Agricultural Research Institute, New Delhi.

All this work on the practical utilization of various induced polyploids would have been impossible without the corresponding theoretical background. It is only thanks to theoretical cytogenetic work that the phenomenon of polyploidy has been discovered and that its great importance to the evolution of plant species has been demonstrated. During this work the difference between auto- and alloploidy has been clarified, and it has been shown that both these categories of variation are involved in the evolution of a great number of our cultivated plants. Under such circumstances attempts to produce new types of polyploids in cultivated plants are well justified, and the results already obtained demonstrate, indeed, that

this method of plant breeding is possible and may lead to quite valuable products.

From what I have said I think it is evident that the classical ways of plant breeding as well as the more recent attempts to utilize mutations and polyploidy have been built up through a close co-operation between theoretical cytogenetics and the experiences of practical breeders and farmers. The theoretical basis is the result of international co-operation and is in the first place founded on Mendel's discovery of stable units of heredity. Especially the Drosophila-workers demonstrated the hereditary function of the chromosomes, and from the same group we have also received the fundamental results on induced mutations and valuable data on the causes of inbreeding degeneration.

Plant breeding based on such premises is certainly not only a theoretical play but a science of the greatest importance to the economy and feeding of the whole world. Besides rational manuring and other agricultural measures consistent breeding work on this basis is the best way so far known to protect humanity from starvation.

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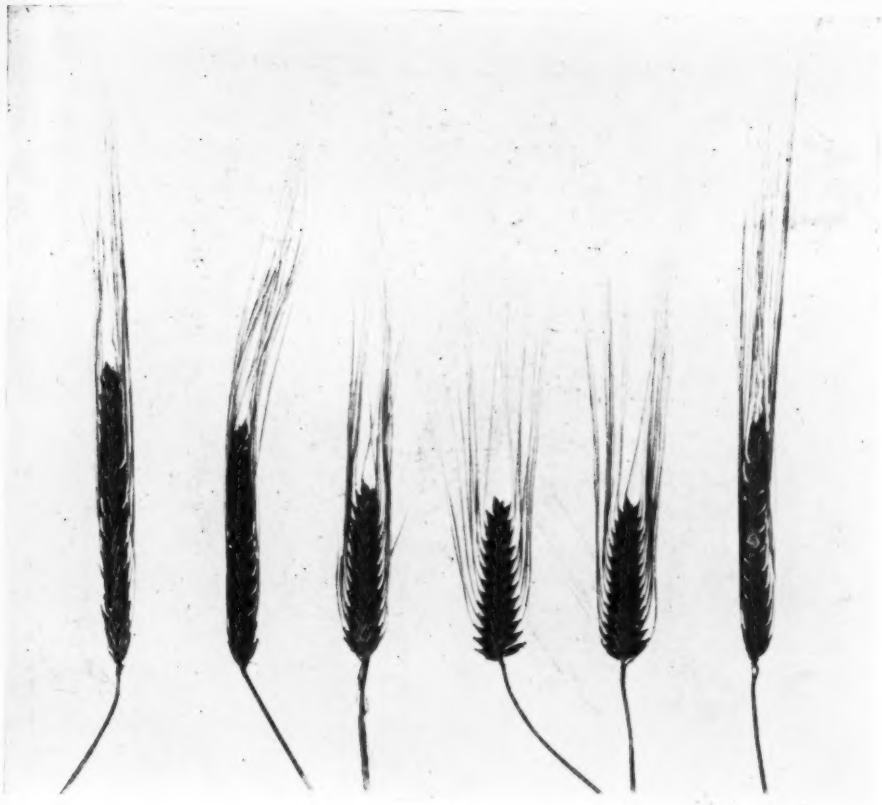

FIG. 1

FIG. 2

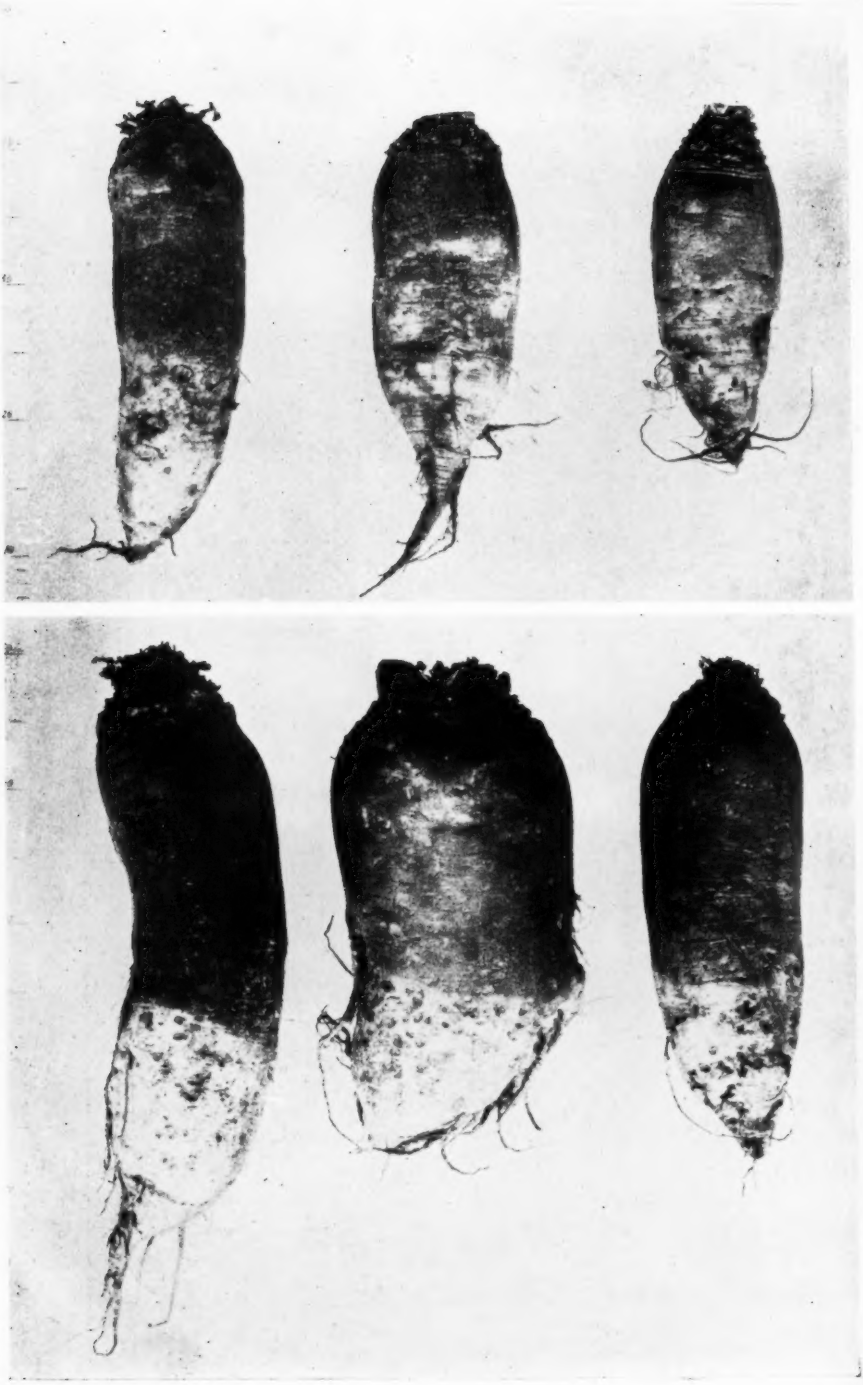

FIG. 3

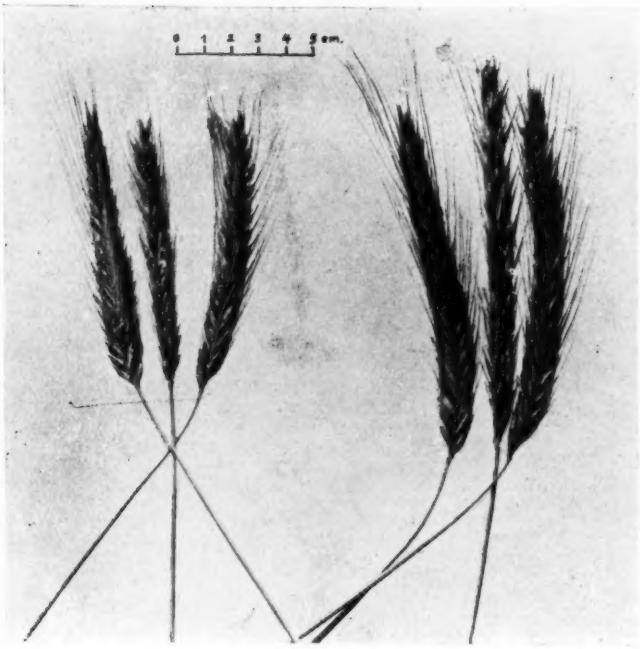

FIG. 4

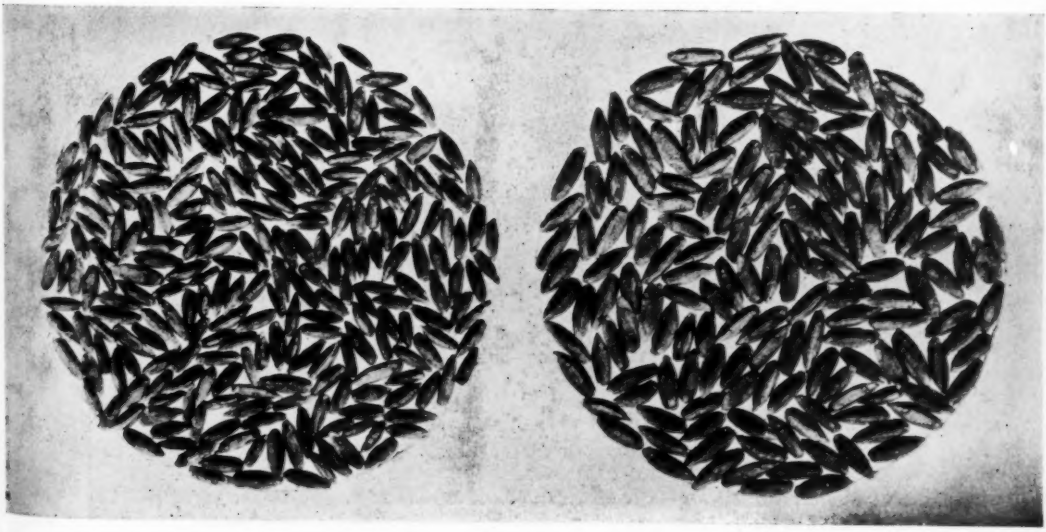

FIG. 5

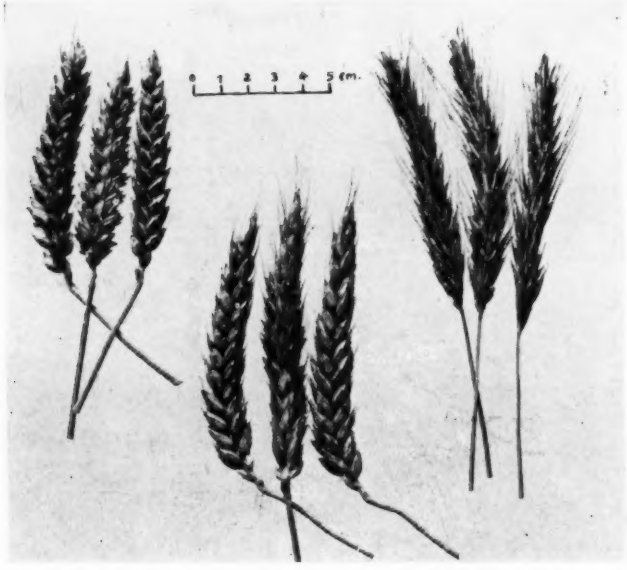

FIG. 6

FIG. 7

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LEGEND

FIG. 1. Maja barley (extreme left) and five different induced mutants from Maja. These mutants are of the so-called *erectoides* type characterized by dense upright ears and a generally increased straw stiffness (Gustafsson and Mac Key, 1948).

FIG. 2. Plots of normal barley (on the right) and an early induced mutation (on the (left) (Gustafsson and Mac Key, 1948).

FIG. 3. Diploid (above) and tetraploid (below) turnip roots of the variety Östersundom. (Levan, 1948).

FIG. 4. Ears of Steelrye, diploid (on the left) and tetraploid (on the right). The scale is 5 cm.

FIG. 5. Kernels of diplold rye (on the left) and of tetraploid rye (on the right).

FIG. 6. Ears of wheat (on the left), a selected inbred strain of rye (on the right) and a Triticale type (in the middle) representing the sum of the chromosome sets of the parents ($42 + 14 = 56$).

FIG. 7. Synthetic winter rape with $20 + 18 = 38$ chromosomes (in the middle) surrounded by the parents, winter turnip rape (on the left) and cabbage (on the right) G. Olsson, unpublished).

FIG. 7

IMMUNOLOGICAL STUDY IN AVIAN MALARIA (*P. GALLINACEUM*) BY THE ANAPHYLACTIC REACTION

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Anaphylactic phenomenon is one of the most remarkable indications of the combination of antigen and antibody. Numerous workers have used anaphylaxis to study the specificity of antigen–antibody reactions [Dakin and Dale (1919)], to determine small amounts of proteins and to disclose differences between antibodies produced in various animal species (Avery and Tillett, 1929; Mehlman and Seegal, 1934). Sufficient evidence has already accumulated to show that, whatever be the exact nature of relation between antigen and antibody, it is dependent up on the stereo-chemical structure of the molecule of the antigen. This observation links up the hitherto puzzling phenomenon of the antigenic disparity between proteins, which to ordinary methods of physical and chemical investigations seemed to be identical. Although corresponding proteins from different species often yield, to an ordinary hydrolytic analysis, the same aminoacids in apparently identical proportions; an immunity reaction such as precipitation or anaphylaxis will discreminate clearly between them. Although the aminoacids are the same however and are present in identical proportions, it is evident that a change in the order of their linkage in the molecule makes possible an immense number of patterns in the structure of proteins. Dakin and Dale (1919) making use of the anaphylactic reaction have demonstrated clearly the differences between crystalline albumins from the eggs of the domestic fowl and the duck. The hæmoglobins of different animal species have also been differentiated by Higashi (1922), Landsteiner and Heidelberger (1923) and Hektoen and Shulhof (1928).

During the course of our studies with *P. gallinaceum* infections in chicken it was necessary to investigate whether the malarial parasite for its growth and multiplication takes up the host cell protein in the original form or in a modified form to suit its requirements. This question arose as several workers (Eaton and Coggeshall, 1939; Zuckerman, 1945) while studying the serological specificity of avian plasmodia using absorption technique

came to the conclusion that the malarial parasite may contain substances antigenically similar to normal erythrocyte components.

In the present investigation attempts have been made to show by biological method whether any difference exists between the hæmoglobin free erythrocyte protein of the chick and the protein of *Plasmodium gallinaceum*.

Methods and Materials

Eaton and Coggeshall (1939) reasoned in order to account for the absorption of antibodies by uninfected erythrocytes, that the parasites cannot be completely separated from the red cells of the monkey or other animals in whose blood they multiply. In order to produce better test antigens a suitable method had to be developed for obtaining malarial parasites free from any fragment of the red cell membrane. By slight modification of the technique devised for freeing *P. lophuræ* (Stauber and Walker, 1946) we were able to free the *Plasmodium gallinaceum* from the infected chicken erythrocytes (Rama Rao, *et al.*, 1951).

Preparation of the Antigens

Antigen No. 1.—Blood from infected chicken were drawn and mixed with heparinised saline buffered to pH 7 to have a concentration of 5 per cent. and centrifuged. Plasma was drawn off, and the cells after washing with buffered saline were suspended in saponin solution (20 mgm. in 100 c.c. of buffered saline pH 7·0). 20 c.c. of saponin solution was used for every 1 c.c. of packed cells and the mixture shaken and centrifuged for 20 minutes. The supernatant solution was drawn off and the cells washed with trypsin solution 0·125 per cent. concentration prepared in buffered saline of pH 5–5·5. After removal of saponin the cells were finally kept in contact with trypsin (for every 1 c.c. of packed cells 10 c.c. of trypsin solution was used) solution for 2½ hours with occasional shaking and the differential digestion was followed by microscopic observation at regular intervals. After 2½ hours the material was centrifuged and washed twice with buffered saline. The brown material consisting chiefly of free malarial parasite and unchanged leucocytes were dispersed in a sterile saline solution after noting the wet weight.

Antigen No. 2.—The above process was repeated with blood from normal chicken. The erythrocyte was treated with saponin to remove hæmoglobin. Further stages were omitted. The packed cells thus obtained after saponin treatment were repeatedly washed with saline and then dispersed in sterile saline after noting the moist weight. Strict aseptic precautions were taken throughout the experiment and the final material was tested for sterility as a routine.

Young guinea pigs of both sexes of uniform weight were chosen for these experiments and were kept in separate cages. The antigens prepared as above mentioned, were diluted with sterile saline to contain approximately 0·1 gm. (wet weight) in 1 c.c. One male and one female guinea pig were used for each of these antigens and 1 c.c. of the antigen was injected intraperitoneally. After intervals varying from 21 to 24 days they were tested for sensitiveness to original antigen in a Dale's bath. The tests were done both on the isolated intestine and isolated uterus by Schultz–Dale technique. During performance of the experiment great attention was given to the use of separate sets of measures, pipettes, etc., to avoid possibility of contaminating one solution with the trace of the other.

Experimental

The sensitised guinea pigs were stunned by a blow on the head and the small intestine and uterine horns were removed. These tissues were kept in warm Ringer solution and allowed to rest for 30 minutes. Strips of uterus or intestine about 3 cms. were then taken and tied with silk thread at each end put up in a Dale's bath to record the movement. The temperature of the bath was adjusted to 37° C. 100 c.c. of Ringer-Locke solution was used for perfusion and oxygen was bubbled at a slow rate. The antigens were added to the bath and the responses obtained were recorded (Figs. I and II).

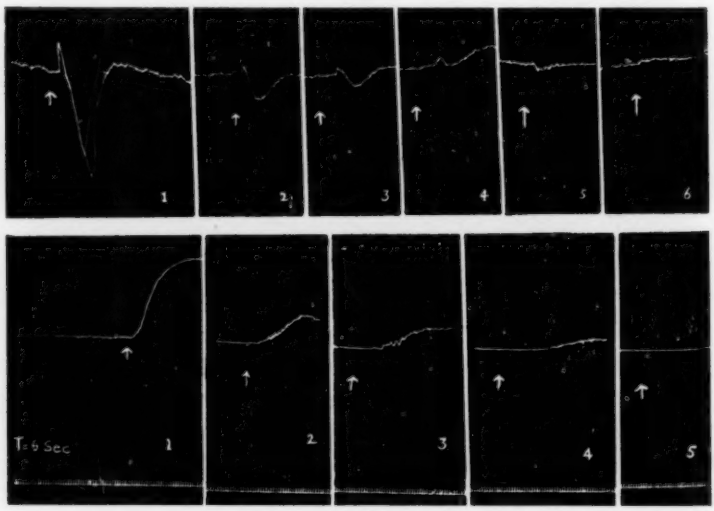

Fig. I

Upper: ♂ guinea pig sensitised with malarial parasite protein, B. wt. 400 g. Time 6 secs. isolated intestine perfused with 1: 1000 suspension of Haemoglobin free protein of

erythrocyte of chick. Note desensitisation is complete in fig. 5. Effect of perfusion with m.p. protein is seen in fig. 6.

Lower: ♀ guinea pig sensitised wirh malarial parasite protein, B.wt. 350 g. Time 6 sees. isolated uterus perfused with 1: 1000 suspension of Haemoglobin free protein erythrocyte of chick. Note desensitisation is complete in fig. 4. Effect of perfusion with m.p. protein is seen in fig. 5. (upward movement of lever denotes contraction).

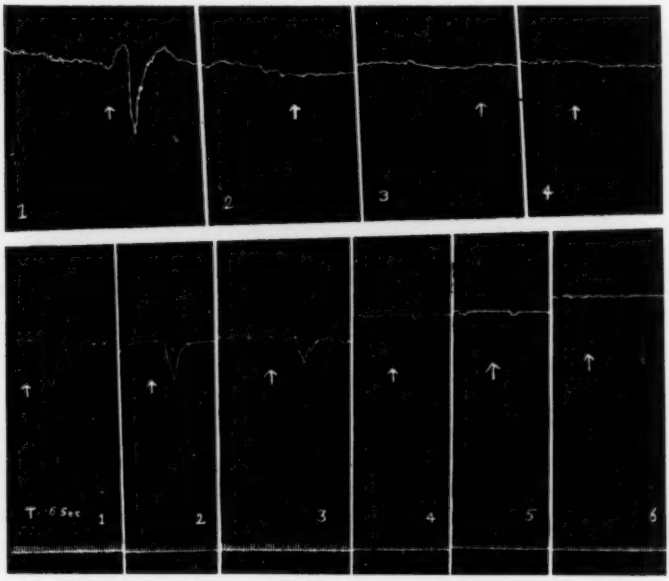

FIG. II

Upper: ♂ guinea pig sensitised with haemoglobin free erythrocyte protein of chick, B. wt. 350 g. Time 6 sees. Isolated intestine perfused with 1: 1000 suspension of malarial parasite protein. Note desensitisation is complete in fig. 3. Effect of perfusing with 1: 1000 normal haemoglobin free erythrocyte protein of chick is seen in fig. 4.

Lower: ♀ guinea pig sensitised with haemoglobin free erythrocyte protein of chick, B. wt. 300 g. Time 6 sees. Isolated uterus perfused with 1: 1000 suspension of malarial parasite protein. Note desensitisation is complete in fig. 5. Effect of perfusing with 1: 1000 suspension of haemoglobin free erythrocyte protein of chick is seen in fig. 6.

ANAPHYLACTIC REACTION

In Experiment No. I male guinea pig weighing 400 gms. and sensitised with malarial parasite protein was used. Strips of intestine showed marked contraction with erythrocytic protein. After repeated injection of erythrocytic protein, the strip was desensitised, and subsequent injection of malarial parasite protein, did not produce any response.

Similar results were obtained with strip of uterus from a female guinea pig sensitised with malarial parasite protein.

In Experiment No. II guinea pigs sensitised with normal erythrocytic protein was used. After desensitisation of intestinal strip with malarial parasite protein, hæmoglobin free erythrocytic protein failed to produce any contraction. Similar experiment with uterine musculature gave identical results. In all cases a first dose of the non-specific antigen caused an apparently maximal contraction. The plain muscles, the uterine or intestine could be completely desensitised to this after repeated doses. After desensitisation with the non-specific protein, the specific protein evoked no response on the part of the muscle.

Discussion

Anphylaxis as a manifestation of the combination of antigen and antibody *in vivo* shows the same degree of specificity as do other antigen–antibody reactions as, for example, the precipitin reaction (Kabat and Mayer, 1948). Schultz-Dale technique is a very delicate and specific test in discriminating between two corresponding proteins. Even slight deviations from the stereochemical pattern of the protein molecule is capable of evoking a different response in animals sensitised with one of the proteins. In the present study, after desensitisation of the smooth muscle with the non-specific protein antigen, preferential sensitisation to the specific antigen was never evoked. This is true both in the case of guinea pigs sensitised with hæmoglobin-free normal erythrocyte protein and with the malarial parasite protein. The result is the same whether the uterine musculature or the intestinal segments were used for evoking the test response. In addition the sensitivity of this test holds good quantitatively even for the amounts of antigen as little as 0·01 mg. for 100 c.c. In our series of experiments the amount of antigen used was of the order of 0·1 gm. per 100 c.c. of perfusing fluid. So far as they go the results once again support the conception of the previous workers that the malarial parasite protein is antigenically similar to the normal protein components of the red blood cells of the host (Heidelberger and Mayer, 1944; Butts, 1945).

Summary

1.. A procedure has been described for preparing hæmoglobin-free erythrocyte protein of the chick and the malarial parasite protein (*P. gallinaceum*) for use as test antigens.

2. Schultz-Dale technique has been used to discriminate between the two proteins and by this procedure they are qualitatively found to be antigenically similar.

This may be one of the reasons to explain why the immunity is not a well-defined phenomenon in malarial infections.

ACKNOWLEDGEMENT

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ON A NEW CALIGID PARASITE, *GLOIOPOTES ZEUGOPTERI*, SP. NOV., FROM *XIPHIAS ZEUGOPTERI*, LAWSON'S BAY, WALTAIR

By T. S. Satyanarayana Rao, M.Sc.

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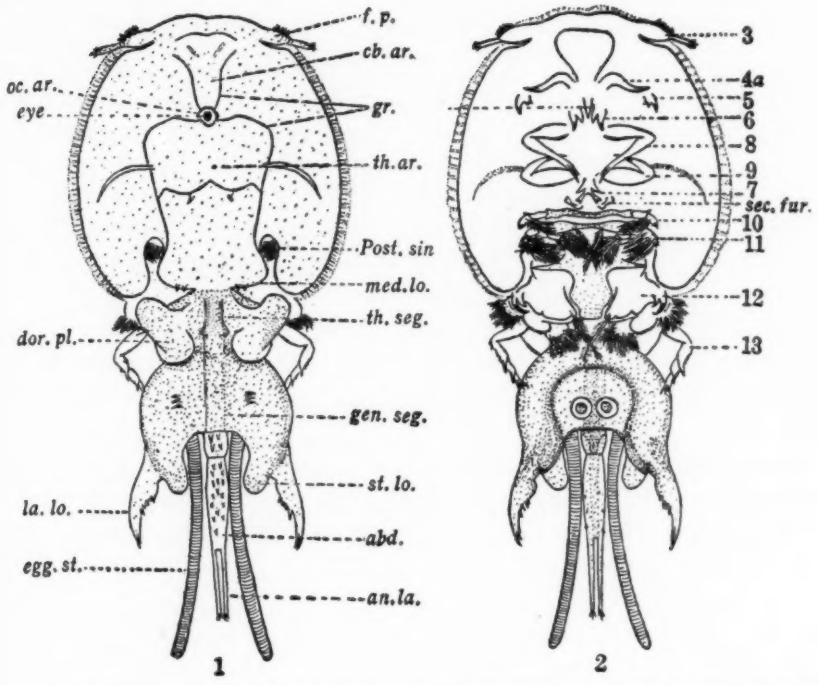
Locality and host.—A number of parasites were collected, both males and females from a sword-fish (*Xiphias zeugopteri*) caught off the coast of Lawson's Bay, in January 1948. They were found attached to the surface of the body and were capable of movement over the body of the fish.

Female (Figs. 1–2).—When fresh, the body is pinkish blue in colour. The egg strings are bright red and protrude from the hinder end of the body. They are approximately one-third the length of the body.

Length of the whole body	9·5 mm.
Length of the carapace	5·5 mm.
Breadth of the carapace	5·0 mm.
Length of the egg strings	3·5 mm.

Carapace.—This is approximately as long as it is broad and is strongly arched laterally. The frontal plates (*f.p.*) are prominent in the anterior region. The carapace is divided posteriorly into a median and two lateral lobes by the presence of two sinuses (*post. sin.*) one on either side. The eye is situated in the middle of the ocular area (*oc. ar.*), which is about one-third of the length of the carapace from the front. This is delimited by the cerebral area (*cb. ar.*) in front and thoracic area (*th. ar.*) behind. The grooves (*gr.*) are arranged in a bilaterally symmetrical manner on the cephalothorax or carapace. From the ocular area start anteriorly two grooves and these proceed towards the bases of the frontal plates. Another pair of grooves start from this area, proceed laterally, then bend posteriorly to finally reach the inner margin of the posterior sinuses. The two grooves are connected by a transverse groove. There are no elevations or depressions on the carapace. The carapace is bordered by a membranous fringe traversed by chitinous striations. There are small setæ arranged all along the margin of the carapace.



abd., Abdomen. *an. la.*, Anal laminæ. *cb. ar.*, Cerebral area. *dor. pl.*, Dorsal plates. *eye.*, The median eye. *egg. str.*, Egg string. *f. p.*, Frontal plate. *gen. seg.*, Genital segment. *gr.*, Grooves. *la. lo.*, Lateral lobe. *med. lo.*, Median lobe. *oc. ar.*, Ocular area. *post. sin.*, Posterior sinus. *sec. fur.*, Secondary furca. *st. lo.*, Stout lobe. *th. ar.*, Thoracic area. *th. seg.*, Thoracic segment.

FIGS. 1–2. Fig. 1. Dorsal view of the female. Fig. 2. Ventral view of the female.

Spines occur mostly on the dorsal surface of the animal. They have a symmetrical arrangement. In the lower half of the thoracic area and beneath the horizontal groove there are two spines. Bordering the free edge of the median lobe are a pair of short spines, on either side. The genital segment (*ge. seg.*) carries three pairs of spines on its dorsal surface. Four spines are present on the first abdominal segment. On the terminal joint and the anal laminæ (*an. la.*) are numerous spines.

Thoracic segment (*th. seg.*).—Immediately behind the carapace is the free thoracic segment. It is broader than long and gives of laterally two dorsal plates (*dor. pl.*) which are longer than broad. These overlap behind a part of the genital segment and also a portion of the basal segment of the fourth

pair of legs. Unlike the male, the female carries no spines on this segment. The dorsal plates are smooth, transparent and pigmented.

Genital segment.—The genital segment (*gen. seg.*) has a narrow neck anteriorly where it joins the free thoracic segment. Behind, it broadens out into more than half the width of the body and is prolonged backwards into a stout lobe (*st. lo.*) on either side of the abdomen. These lobes curve inward and toward the abdomen. Externally a pair of lateral lobes (*la. lo.*) are given off. Their inner margins and tips are provided with spines. The dorsal surface of the genital segment carries a few spines arranged symmetrically, some distance from the middle line.

Abdomen.—Abdomen is two jointed, the basal joint is wider and shorter than the terminal joint. The anal laminæ do not end in plumose setæ as in other forms of the genus *Gloiopotes*, but in small spines which are present at the tips.

Appendages: 1*st Antennæ* (Fig. 3).—These are prominent. Each is three jointed, the terminal being longer and narrower than the basal joint. The middle joint has plumose setæ on its outer border. The distal segment bears a tuft of spines at its distal end.

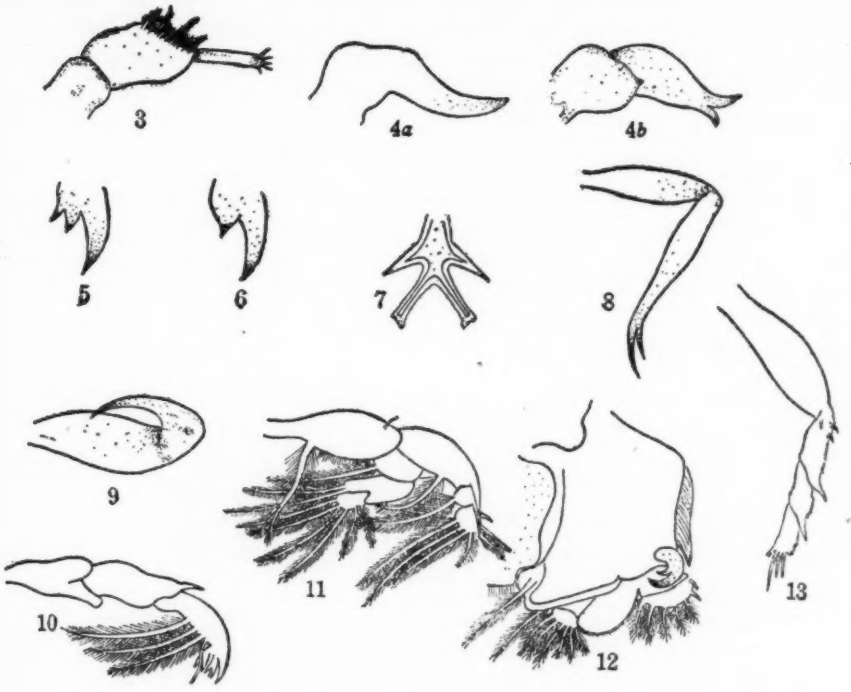
2*nd Antennæ* (Fig. 4 *a*).—They are smaller than the first antennæ, and each is two jointed; the basal segment shorter and broader than the narrow distal segment which tapers in a strong claw. No accessory setæ are present.

1*st Maxillæ* (Fig. 5).—These are situated behind and below the second antennæ and each is three parted. In each maxilla the outer spine is very large when compared with the inner spines which are sub-equal.

2*nd Maxillæ* (Fig. 6).—Each is short and stout, bifurcated for more than half its length and the outer branch is longer than the inner. The spines are very chitinous and turn dark brown in alcohol.

Furca (Fig. 7).—It is present on the median axis of the body below the maxillipeds. It is a compound structure with branches between which lie a broad 'U'-shaped sinus. Each inner branch is at the base divided into two short and blunt knobs. These turn brown in alcohol. There are also a pair of secondary furca (*sec. fur.*) on either side of the primary one described above.

1*st Maxillipeds* (Fig. 8).—Each is two jointed and slender; the basal joint is shorter and stouter than the terminal joint which ends in small spines.



FIGS. 3–13. Fig. 3. 1st Antenna. Fig. 4 *a*. Second antenna of the female. Fig. 4 *b*. Second antenna of the male. Fig. 5. 1st Maxilla. Fig. 6. 2nd Maxilla. Fig. 7. Furca. Fig. 8. 1st Maxilliped. Fig. 9. 2nd Maxilliped. Fig. 10. 1st Swimming leg. Fig. 11. 2nd Swimming leg. Fig. 12. 3rd Swimming leg. Fig. 13. 4th Swimming leg.

2nd Maxillipeds (Fig. 9).—These are shorter and stouter than the first. In each maxilliped the basal segment is huge and the second segment ends in a strong claw. There are no accessory spines over it.

1st pair of swimming legs (Fig. 10).—These are uniramous; three segmented; the members of the pair are interconnected by a sternal rod as in the case of Caligidæ in general. A spine is present on the distal segment at the outer margin. The most characteristic feature is the presence of two posteriorly directed tripartite spines on the inner margin of the distal segment. There are three plumose setæ and an extra seta on it.

2nd pair of swimming legs (Fig. 11).—These are biramous; sternum is present; each exopod or endopod consists of three joints and all possess plumose setæ.

3rd pair of swimming legs (Fig. 12).—The basal plate of the third leg is very broad and triangular. The exopods and endopods can be compared to those of the second pair of legs. The rami bear plumose setæ as well as spines. Members of the pair are interconnected by a broad plate which is speckled like the dorsal plates.

4th pair of swimming legs (Fig. 13).—These are uniramous; no sternum; basal segment is larger and stouter than the terminal segments which are three in number and distinctly separated from one another. The basal segment has no spines. Each terminal segment bears a large spine at its distal end, and also a fringe of small teeth on the outer margin behind the larger spine.

Details of Spine arrangement:

1st legs: Uniramose

2nd segment	..	one spine.
3rd segment	..	one spine, two tripartite spines and three plumose setæ and one seta.

2nd legs: Biramose

Basal segment	..	one spine and one plumose seta.
Exopod		
1st segment	..	one spine and one plumose seta.
2nd segment	..	one spine and one plumose seta.
3rd segment	..	one spine and six plumose setæ.
Endopod		
1st segment	..	one plumose seta.
2nd segment	..	two plumose setæ.
3rd segment	..	six plumose setæ.

3rd legs: Biramose

Basal segment	..	one plumose seta.
Exopod		
1st segment	..	one spine (bifid).
2nd segment	..	two spines and five plumose setæ.
Endopod		
2nd segment	..	one plumose seta.
3rd segment	..	six plumose setæ.

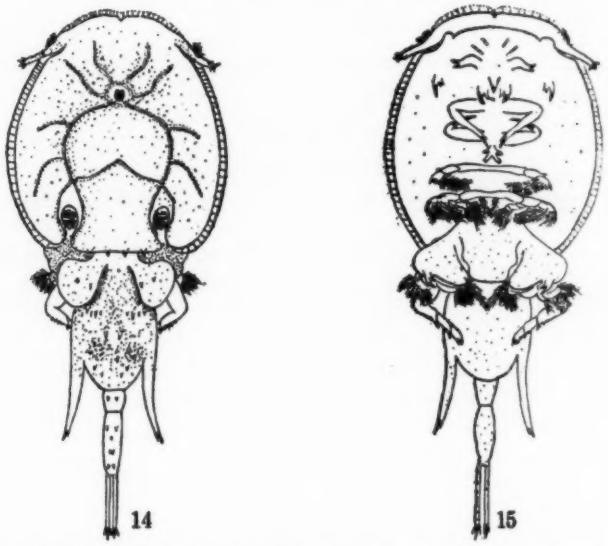
4th legs: Uniramose

All the three distal segments bear spines on their outer margin.

The genital apertures are situated at the base of the genital segment on either side of the abdomen. Through these the egg strings project out.

These are uniseriate and consist of variable number of eggs. Instances where the lengths of the egg strings being different on either side are frequently noticed. On the ventral side of the genital segment there are two circular depressions in which are lodged two hemispherical structures and these appear to be formed out of agglutination of fine chitinous threads. The function is not known.

Male (Figs. 14–15).—Males are smaller than the females. There is resemblance between the male and the female in general organisation but there



FIGS. 14–15. Fig. 14. Dorsal view of the male. Fig. 15. Ventral view of the male. Names of parts same as given for female.

are many differences in other features. The important points of differences are as follows:

1. Body shape generally broader.
2. The second antenna has an accessory claw on the outer margin behind the main claw (Fig. 4 *b*).
3. The genital segment is not horse-shoe-shaped. It does not give off posteriorly the two inner stout lobes as in the case of the female. The lateral lobes are however present, but are slender.

Systematic position.—This form belongs to the *Caligidæ* and the subfamily *Euryphorinæ* as the following characters show:

1. Carapace is broad and compressed and is made up of three anterior segments fused with the head.
2. Frontal plates are well defined.
3. The fourth thoracic segment is small, free and furnished in the case of both males and females with a pair of dorsal plates which usually overlap the genital segment.
4. Abdomen is two jointed and elongated.

The present form belongs to the genus *Gloiopotes* because of the following characters:

1. The three thoracic segments being fused with the head, the fourth segment being free.
2. One or more pairs of legs uniramose.
3. First and the fourth legs are uniramose; claws on the first pair straight and three parted; setæ on anal laminæ long and plumose.

KEY TO THE GENUS *Gloiopotes* STEENSTRUP AND LUTKEN

1. Dorsal plates on the IV segment short and rounded covering only a little of the genital segment ..... 2.
1. Dorsal plates elongate, rectangular, covering all the genital segment except the processes ..... *hygomianus* St. and L., 1860.
2. Dorsal plates semilunate, longer than wide; first antennæ concealed; abdomen smooth ..... *huttoni Thomson*, 1889.
2. Dorsal plates much wider than long; first antennæ prominent; abdomen heavily armed with spines along the sides ..... *ornatus Wilson*, 1905.

Since then Kirtisinghe has described the species *Gloiopotes watsoni* (1933). The characters of this form are that the dorsal plates are kidney-shaped and provided with spines; the anal laminæ carry plumose setæ; the posterior claws of the first legs are bifurcate; the outer ramus of the third leg is armed with a three-pronged claw. The present form differs from the above species in:

1. the shape of the dorsal plates which are longer than broad,
2. the posterior claws of the first legs being trifurcate,
3. the outer ramus of the third leg being armed with bifid claw, and finally,
4. the anal laminæ not bearing any plumose setæ.

The present form also differs from *G. hygomianus* St. and L., in the dorsal plates not covering all the genital segment and from *G. huttoni* Thomson in not having a concealed antennæ and by possessing abdomen armed with spines. Finally this form shows variation from *Gloiopotes ornatus* Wilson, in the shape of the dorsal plates and in their being longer than broad and in the presence of spines on the ventral and dorsal sides of the abdomen.

It will thus be seen that the present form cannot be said to belong to any of the species described under the genus *Gloiopotes* and therefore it is described as a new species, *Gloiopotes zeugopteri* sp. Type specimen is kept in the Andhra University Zoological Laboratory.

Acknowledgments

In conclusion, I wish to thank Professor R. Gopala Aiyar for the constant help, encouragement and advice that I have received in the preparation of this paper. This work was carried out in the Zoological Laboratories of the Andhra University, Waltair.

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A PHOTOGRAPHIC METHOD FOR DETERMINING APICAL GROWTH IN FUNGAL HYPHÆ

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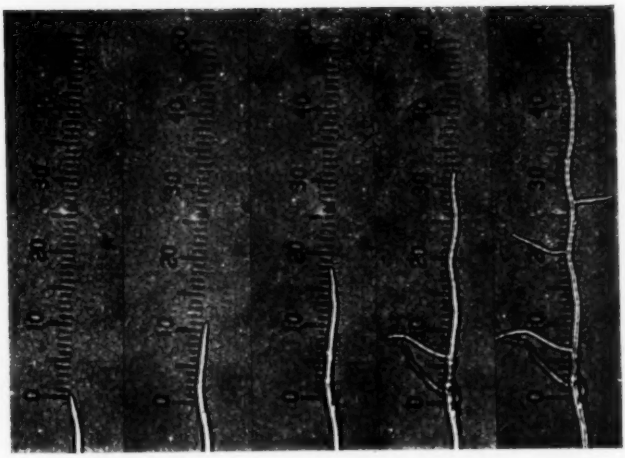
It is well recognized that growth in fungal hyphæ is only apical and considerable evidence to this effect has been adduced by Smith (1923) in the case of a variety of fungal forms. The technique developed by him of sowing fungal spores on a cover slip coated with agar medium and inverting the latter on a van Tieghem cell, growth measurements being made at intervals of time, has obvious shortcomings in its applicability to measure growth rate of rapidly growing fungi; the fastest growth obtained by Smith (1923) in the case of *Rhizopus nigricans* was less than 100 microns per hour which is too low a figure for a rapidly growing fungus like *R. nigricans*; it is not clear whether this was due to the low nutrient content of the medium, or the influences of the van Tieghem cell, or due to the intrinsic nature of that particular isolate itself. Nonetheless the limitations of van Tieghem cell and its tendencies to exaggerate anaerobic influences on fungal growth are evident. The present technique was developed to measure growth rate *in vitro* aiming chiefly at providing tangible evidence of apical growth in fungal hyphæ for Laboratory demonstration purposes, on somewhat similar pattern to the photographic method described by Vyvyan (1924) for measuring growth rates of leaves. As far as the author is aware no photographic evidence is available of the microscopic growth of fungal hyphæ and its complementary changes during growth (branching, etc.).

Rhizopus nodosus, a very fast growing strain isolated from cotton seed by the author (Venkatram, 1950), was employed in this investigation. The fungus was inoculated in the centre of sterile agar plates of standard potato dextrose medium; 10 cm. 'K' Petri dishes were selected for this purpose since they were convenient to handle, comparatively shorter and of good transparency to facilitate direct microscopic observation; the agar medium was filtered thrice to obtain good clarity. Growth measurements were made six hours after inoculation using Zeiss × 10 objective and screw type adjustable eyepiece micrometer. One single hypha was brought into the



FIGS. A–E show growing hyphæ of *Rhizopus nodosus* (×60). The time interval between exposures *seriatim* from A to B to C to D to E was 2½, 1½, 3½ and 4 minutes respectively.

microscopic field so that the hyphal tip came flush with the 0 scale of the micrometer; thereafter the number of divisions traversed after suitable intervals of time were noted or *vice versa* the time taken to cover the distance of 5 micrometer divisions was determined by a stop-clock arrangement. Due to the very rapid growth of the fungus (in this case being nearly 60 microns per minute), accurate camera lucida drawings of the branching habits, etc., were not possible, consequently photomicrographs of the growth of the fungal hyphæ were taken at suitable intervals of time using a Zeiss Miflex camera attachment. This arrangement enabled correct focussing of the fungal hyphæ at different stages of growth, to be achieved, just prior to the photographing. Illumination of the microscopic field was so manipulated as to give a good picture with exposure of one second on Ilford H.P. 3 Plates. Very satisfactory results were obtained by this method as is seen from Plate XVI.

It was observed that although branching occurred freely, the position of these branches on the micrometer scale never changed (Plate XVI), thus proving that hyphal growth was entirely apical. Since the conditions provided herein were comparable to those for determining the radial growth of fungi in any desired medium, rate of growth measured by this technique would serve as a suitable index to compare growth rates between fungal forms in a definite medium or inversely of one form in different media, at a particular time. In actual practice it was found that figures obtained in microns of the growing hyphal tip converted into mm. then multiplied by 2 (factor 2 gives diametrical spread in two directions being a colony) and calculated against time approximately gives colony diameter in mm. which is the conventional unit of growth measurement of growing fungi in culture media.

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THE EFFECT OF A SINGLE INJECTION OF THYROXIN ON THE PROTEIN AND NUCLEIC ACIDS OF THE MOUSE LIVER AND THE PROTEIN OF THE MOUSE CARCASS

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The role of the thyroid in the mobilisation of liver nucleic acids and protein in the rat has been demonstrated by several workers (Canzanelli, Rapport, and Guild, 1949; Frankel-Conrat and Li, 1949: Drabkin, 1950). These investigations involve prolonged and repeated administration of the thyroid material to rats and sacrifice of the animals at an arbitrarily chosen time. Clinical hyperthyroidism (Lichtman, 1941) is frequently accompanied by liver damage. It is conceivable that in the rat as well, hyperthyroidism produced by prolonged administration of thyroid material may lead to liver damage and exacerbation of thyrotoxic symptoms due to the inability of the damaged liver to metabolise thyroxin (Grad and Leblond, 1950). This probably explains the observation of Drabkin (1950) on the pronounced impairment of the regenerative process of the liver in hyperthyroid animals. It has been convincingly shown by Gaddum (1930) and Sternheimer (1939) that the effect of a single dose of thyroxin on the oxygen consumption of tissues and chemical changes in the liver is not uniform throughout, but varies according to the time elapsed after injection. Recently Wallach and Reineke (1949) have emphasised the importance of time relationship in the response of the adrenal to prolonged thyroid stimulation. With the above observations in view, experiments are in progress in this Laboratory to study the effect of varying levels of single dose of thyroxine on the mobilisation of liver nucleic acids and protein in the mouse and the role of the adrenal and pituitary in such processes. The present paper records the results of initial observations.

Experimental

Inbred male mice of the C-57 strain varying in age from six to seven months, raised on the stock diet (Talageri, *et al.*, 1951) were used. The experimental group of animals were injected subcutaneously through the loose skin of the back with a weakly alkaline solution of DL-thyroxin (Squibb)

at a level of 4 mg. per kg. body weight, while the controls received an equal volume of normal saline. The animals were fed *ad libitum* and sacrificed at definite intervals by sturning. In general, all the animals were fasted for a period of 14-16 hours before sacrifice, except the animals of Group II which were fasted only for 6 hours, limited by the experimental conditions. Control mice fasted for a similar number of hours as those of Group II showed no significant variation in the liver protein and nucleic acids from those fasted for 14–16 hours. Two control animals were always sacrificed together with the treated animals. The livers were removed *in toto* and weighed on a Roller-Smith torsion balance. The liver was homogenised in cold water and diluted to contain 10 per cent. by weight of tissue. Aliquots of the homogenate were used for the estimation of total nitrogen according to the method of Sobel, Yuska and Cohen (1937). The nucleic acids were estimated according to the method of Schmidt and Thannhauser (1945) modelled after Schneider (1946) on 4 ml. aliquots of the homogenate. Phosphorus was determined by the method of Fiske and Subbarow (1925) as modified by LePage and Umbreit (1945). In some experiments, total nitrogen on the carcass (the carcass consisted of the entire animal *minus* liver) and total liver were determined by the conventional macro-Kjeldahl method. In all these experiments, total nitrogen of the tissues is assumed to be a measure of the total protein.

Results

As demonstrated in Table I, it is apparent that there is no change in total nitrogen of the mouse liver during the ' latent period ' (Gaddum, 1930) following the administration of a single dose of thyroxin. However, at the end of 48 hours, the total nitrogen content of the liver begins to rise reaching a maximum value, 96 hours after the administration of the drug. Thus the difference between the total nitrogen values of Groups I and V are highly significant. At the end of 120 hours, there is a precipitous fall in the total nitrogen of the liver, this value being not significantly different from the controls. Sternheimer observed two peaks for total nitrogen of the rat liver at the end of 60 and 144 hours respectively, under identical experimental conditions. Since there is a maximum increase in total nitrogen of the liver, at the end of 96 hours in the treated animals, the relationship of the carcass nitrogen to the increase in liver nitrogen at this time was investigated. The results are given in Table II. It is evident from the figures that there is a 5 per cent. loss of nitrogen from the carcass per 100 gm. initial body weight in the treated animals.

The changes in the RNA and DNA of the liver after a single injection thyroxin are interesting. At the end of six hours, there is a marked fall of

TABLE I

Changes in Nucleic Acids and Total Nitrogen of the Mouse Liver after a Single Subcutaneous Administration of DL-Thyroxin

Group No.	No. of mice	Time after injection	Nucleic acids		Total nitrogen
			RNA	DNA	g./100 g. wet weight
			mg. P/100 g. wet weight		
I	10	Control	93·0±2·2 (20)	25·5±0·7 (20)	2·87±0·06 (20)
II	3	6 hr.	73·4±1·3 (6)	23·4±0·7 (6)	2·97±0·01 (6)
III	5	18 ,,	120·6±3·1 (10)	24·5±1·0 (10)	2·93±0·03 (12)
IV	9	48 ,,	118·5±3·7 (12)	21·2±1·4 (12)	3·45±0·10 (12)
V	6	96 ,,	125·0±2·3 (12)	30·4±1·2 (12)	3·72±0·19 (10)
VI	7	120 ,,	122·7±2·4 (12)	24·7±1·0 (12)	2·97±0·03 (11)
VII	5	120 ,,	114·1±0·9 (10)	26·8±0·6 (10)	2·97±0·04 (10)
VIII	5	240 ,,	100·4±1·7 (10)	25·1±1·7 (10)	2·96±0·04 (10)

The figures in the parenthesis below each column indicate the number of estimations. The measure of variability is the standard error of the mean. In Tables I and II, all values are means and standard errors. The standard errors have been calculated using the formula, $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

TABLE II

Changes in Total Nitrogen in the Liver and Carcass after a Single Subcutaneous Administration of DL-Thyroxin in Mice

Group No.	No. of mice	Time after injection	Liver nitrogen	Carcass nitrogen
			mg.	mg.
I	5	Control	121·0±4·4	2·82±0·04
V	5	96 hr.	*142·8±6·2	2·68±0·05

All nitrogen values are expressed in terms of 100 gm. of initial body weight. * Represents values of four animals only.

the RNA which appears to rise very fast reaching a maximum value in eighteen hours following administration of the drug. From this time onwards, it almost remains steady at this high level till the end of 120 hours after which

TABLE III

Comparison of the Alteration in Liver Nucleic Acids and Protein and Carcass Protein as Influenced by the Administration of Single Injection of DL-Thyroxin

Groups compared	Liver Nucleic Acids		Liver nitrogen	Carcass nitrogen
	RNA	DNA		
I *vs.* II ..	−19·6±2·6	..	..	..
I *vs.* III ..	+27·6±3·8	..	..	..
I *vs.* IV ..	..	−4·3±1·6	..	..
I *vs.* V ..	+32·0±3·2	−4·9±1·7	+ 0·85±0·2 +21·8*±7·5	−0·14±0·06
V *vs.* VI	..	+9·2±1·8	..	..

The values in this table are arithmetical differences of means and standard errors of the differences obtained by comparison of the data in Table I and II for the indicated groups of animals. The standard errors of the differences were calculated from the standard error of the means in Tables I and II using the expression, standard error of differences $= \sqrt{(SE_1)^2+(SE_2)^2}$. The differences of the means were considered significant when they exceeded by at least three-fold the standard errors of the differences. * Data derived from Table II.

RNA concentration levels off close to the control values. The values of RNA in Groups III to VI compare favourably with the figures of Canzanelli, *et al.* (1949) for the prelobectomised rat liver after feeding dry thyroid at a level of 500 mg. per day for ten days. It is generally considered that the DNA concentration in the somatic tissues is notoriously constant (Boivin, Vendrely and Vendrely, 1948, Mirsky and Ris, 1949). But there is also the contrary evidence that the DNA content of nuclei and chromosomes is variable in different tissues (Schrader and Leuchtenbergear, 1949). This may be accounted for in one of two ways: (i) That DNA has no precise quantitative relation to the basic structure of the chromosomes and may be variable depending upon the age or physiological conditions of the nucleus or its *milieu*. In consequence an accumulation or depletion of nucleic acid charge may be observed. In this sense cellular activity, nucleic acid metabolism and nuclear structure become intimately related (Darlington and La Cour, 1940; Darlington, 1942). (ii) A second possibility based on the fundamental cytological concept that a chromosome consists of strands or chromonemata which may be single, double or multiple (polytene). If each of these strands is charged with a definite and identical quantity of DNA, then the quantitative relationship of DNA in a chromosome would b determined by the constituent number of chromonemata. As Schrader and

Leuchtenberger state, "a chromosome of a high degree of polyteny would therefore contain more DNA than an otherwise homologous chromosome of a lower degree of polyteny". Both these cytological concepts have a measure of validity. It is true that there are certain flaws in the argument of Schrader and Leuchtenberger as pointed out by Ris and Mirsky (1949); but the general occurrence of polysomaty and polyploid sectors in obviously diploid tissues is now increasingly becoming recognized as shown by the reports of Geitler (1938), Huskins, *et al.* (1947) and Huskins and Steinitz (1948). Moreover, as Davidson and Leslie have pointed out (1950), if the constancy of the DNA of a nucleus is causally connected with chromosome number, *i.e.*, to the 'ploidy' of the nucleus, the DNA content of the sperm should be the same as that of the egg (as they contain the haploid complement of the chromosomes) and half of that of the somatic nuclei. But this is not the case (Davidson and Leslie, 1950). There are also the findings of Chargaff and Saidel (1949) and Vischer, Zamenhoff and Chargaff (1949) that the composition of DNA is not always the same and may provide a basis of variation. In addition, the researches of Pollister and Leuchtenberger (1949) have furnished evidence that physical differences depending on diverse degrees of polymerization could account for the variation of DNA. Even in the biochemical work of Mirsky and Ris (1949), it should be noted in passing that the DNA values of particular tissues differ from those given by Boivin, Vendrely and Vendrely for the same tissues. In view of the foregoing, more extensive investigations are necessary before making an unqualified acceptance of the thesis of DNA constancy. The thyroxin work reported here manifestly substantiates this. However, as seen from the results in Table I, there are some changes in the DNA concentration following the administration of a single dose of thyroxin. At the end of 48 hours the DNA concentration has fallen down and this value compared to the control is just significant. At the end of 96 hours, the DNA has climbed to a maximum value and levels off with the control value in 120 hours from the start of the experiment. The difference in the DNA values between Groups IV and V are very highly significant. Changes in the DNA concentration do not seem to take place till the values of RNA have reached a maximum value. Incidentally, it may be pointed out that the values for the total nitrogen of the liver and DNA reach the peak level just about the same time. Canzanelli and co-workers (1949) have observed that the concentration of the liver DNA is affected to "some extent" by thyroid hormone. The present report bears out this observation in a more significant fashion. How far the biochemical picture presented here is reflected in the cytological and histochemical topography of the liver tissue will be dealt with in a

separate communication by Shanta V. Iyengar and Gopal Ayengar of this Laboratory.

Discussion

It must first be pointed out that the dosage of thyroxin administered in these experiments is several times above the normal secretion of the mouse thyroid per diem. Whether or not, the present results apply to normally secreting glands, they have a bearing in cases in which there is a stimulation of the metabolism by thyroxin, as for example, in Graves' disease. Chemical studies have indicated that liver cell can either excrete thyroxin into the bile (Kendall, 1919; Barnes, 1933) or inactivate it (Elmer, 1938). More recent experiments with labelled thyroxin (Gross and Leblond, 1950; Clayton, *et al.*, 1950) have suggested that both processes could take place under physiological conditions. Particularly the experiments of Gross and Leblond (1950) have indicated that a major fraction (nearly 60 per cent.) of large doses of thyroxin administered to rats is excreted in the urine and feces in 72 hours. Gaddum (1930) has demonstrated three distinct phases following administration of a single dose of thyroxin at a level of 4 mg. per kg. of body weight. The three phases comprise, (*a*) " latent period " for 18 hours, (*b*) a period of active metabolism as indicated by increased oxygen uptake lasting for 3–9 days and (*c*) return to normal conditions. The results in our experiments presented in Tables I and II clearly indicate that a majority of the changes take place during a period of increased metabolism; the only exception being the marked fall of RNA six hours after injection of the drug. Since most of the changes observed in this investigation take place at a time when a major fraction of thyroxin could be conceived to have been excreted, it seems profitable to conclude that the observed metabolic stimulation is the end result of a series of chain reactions initiated while a major fraction of thyroxin was present in the body.

Sternheimer (1939) has pointed out that the administration of a single dose of thyroxin to rats seems to have no significant effect on the liver non-protein, amino acid and ammonia nitrogen. Therefore the observed increase (18 per cent. per 100 gm. initial body weight) of the total nitrogen of the liver in Group V may be assumed to be essentially due to an increase in liver protein (Table II). Increase in total protein content of the liver after repeated injections of thyroxin has been reported even when the animals were fasted (Addis, *et al.*, 1938). The loss (5 per cent. per 100 gm. initial body weight) of carcass protein observed in Group V (Table II) cannot completely account for the total increase in the liver protein, which in part is probably derived from convertible carbohydrate sources as well. White and Douhgerty (1947)

who studied the effect of endocrine secretions on the loss of protein during fasting have suggested that the mobilisation of nitrogen from the carcass is under thyroid control.

As has already been pointed out there is a sudden fall in the liver RNA in 6 hours after treatment with thyroxin. Whether this fall reflects on the inhibitory action of thyroxin on liver cellular activity or otherwise cannot be substantiated at this stage.

There is a significant mobilisation of liver RNA in the thyroxin-treated animals during the entire phase of active metabolism (18–120 hours after the administration of thyroxin). Sternheimer (1939) has presented histological observations of the rat liver following the administration of thyroxin. These indicate that thyroxin induces active growth during a period of increased metabolism with changes in the size and structure of the liver cells similar to those observed in the regenerating liver. Further, the experiments of Canzanelli, *et al.* (1949) point to a control of liver regeneration in the rat by the thyroid. According to Novikoff and Potter (1948), the RNA content is greatest during the period of most active regeneration. On the other hand, on the basis of evidence presented by Friedkin and Lehninger (1949) on the participation of mitochondrial RNA of rat liver in oxidation over the Krebs tricarboxylic acid cycle, it seems to us that the necessary association of the active growth with RNA mobilisation must be taken with some reserve. The existence of a correlation between the apparent mobilisation of liver protein and nucleic acids in the results presented above must also be recorded with the same reserve, particularly in view of the observations of Spiegelman and Kamen (1947) on the possible role of nucleoproteins in protein synthesise All the above facts have been recorded without prejudice or postulate.

At this stage of our investigation we are unable to offer any explanation for the apparent changes in the liver DNA following treatment with thyroxin.

Summary

1. Observations have been made concerning the effect of a single dose of thyroxin at a level of 4 mg. per kg. of body weight on the changes in the liver protein and nucleic acids at definite intervals in C-57 strain of mice.

2. Except for a marked drop in the liver RNA 6 hours after thyroxin administration, mobilisation of liver protein and nucleic acids take place during a period of active metabolism (18–120 hours after thyroxin administration). Some clear changes in the liver DNA have been also observed during the period of active metabolism.

3. There appears to be no quantitative correlation between the mobilisation of protein in the liver and loss of carcass protein, 96 hours after the administration of thyroxin.

4. The significance of the observed changes has been presented without prejudice or postulate.

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EXCRETION OF VITAMIN C DURING GERMINATION OF LEGUMINOUS SEEDS

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Germination of seeds is generally preceded by a process of steeping in flowing water which serves to soften the cell walls and equalise the moisture of the tissues. It is familiar knowledge that the process of germination is attended by a variety of changes, mostly enzymic in nature; in fact, germinating seeds show increase in the activity of most enzymes. As a result, there is a considerable breakdown of the proteins into simpler peptides and amino acids, a partial saccharification of the starch and a release of inorganic phosphates from the phytin.[1, 2] The biochemical changes that occur during germination are typified by the studies on the chemistry of malting.[3]

The elaboration of vitamin C during germination is related to the several other biochemical transformations taking place during sprouting.[4] Taking into account the high solubility as well as extreme lability of ascorbic acid and considering that during the steeping and subsequent sprouting of seeds, their moisture content varies from 60–90 per cent., it appeared worthwhile to study whether, in addition to the elaboration of the vitamin in the germinated seed, a part of it was also excreted into the surrounding medium. It is possible that such an observation has not been recorded by earlier workers because of the high susceptibility of the vitamin to rapid oxidation to dehydroascorbic acid and, irreversibly, to diketogulonic acid.

In studies on the excretion of amino acids by legumes, Virtanen and co-workers[5–9] have shown that a considerable part of the nitrogen fixed by root nodule organisms is secreted chiefly as aspartic acid and as degraded β-alanine; this secretion of nitrogen is favoured in presence of media which could absorb the excreted nitrogen. The old established fact that non-legumes benefit by an association with legumes, is explained on this basis; in fact these authors observed an increased excretion of nitrogen in associated cultures of inoculated legumes and non-legumes capable of utilizing the excreted nitrogen compounds.

Experimental

Preliminary overnight soaking of the seeds in water was done either in beakers or in Petri-dishes; subsequent germination was allowed to proceed by spreading out the soaked seeds in Petri-dishes lined with moistened

cotton wads. In order to provide for prevention of auto-oxidation of any ascorbic acid that might be excreted either in the steep water or during germination, recourse was had to the use of thiourea and sulphur dioxide, the latter as potassium meta-bisulphite. These compounds are known to stabilize aqueous ascorbic acid against oxidation.[11-12] It was observed, however, that thiourea, even in concentrations as low as 500 p.p.m., inhibited germination; hence, the studies reported here relate only to the use of potassium meta-bi-sulphite in a concentration of 300 p.p.m.; at this concentration, germination was apparently normal and comparable to control lots steeped in or treated with water alone.

Ascorbic acid and total ascorbic acid (ascorbic acid *plus* dehydro-ascorbic acid) determinations were carried out both in the steep water used during preliminary soaking and in the moist cotton wads at different intervals during germination. Mapson's procedure[13] for the determination of the vitamin in presence of sulphites, sulphides and thiol compounds was adopted; from recovery experiments, it was always possible to secure an accuracy within 2–3 per cent.

For germination in the dishes, the cotton wads were kept moistened with water containing 300 p.p.m. of potassium meta-bi-sulphite. In the examination of cotton wads for the presence of ascorbic acid during various stages of germination, the wads were first drained out of the absorbed meta-bi-sulphite solution, extracted at least twice with the least quantity of water containing potassium meta-bi-sulphite and made up to volume. An aliquot of the total extract was made to 4 per cent. strength of oxalic acid for the determination of ascorbic acid and total ascorbic acid.[4] Results represent averages of triplicate determinations.

Results

When *mung* (*Phaseolus radiatus*) seeds were soaked overnight in 5-6 times water, it was observed that the steep water did not contain any ascorbic acid or dehydro-ascorbic acid, although the ascorbic acid content of the soaked seeds had risen from nil to 40 mgm. per cent. on dry basis. On the other hand, if instead of water, a solution of potassium meta-bi-sulphite (300 p.p.m.) was used for steeping, ascorbic acid could always be detected in the steep water. Thus, using 10 gm. lots of *mung* seeds, the results obtained after varying periods of soaking are given in Table I together with similar results for *Masoor* (*Lens esculenta*) and black pea (*Pisum arvense*); with the latter sets, the solutions in which the seeds were soaked turned pink on acidification and hence a modified procedure for coloured extracts[14] was followed.

TABLE I

Ascorbic acid excretion during steeping

Legume	Period of soaking (in hours)	Excreted ascorbic acid (in mg.)	Excreted total ascorbic acid (in mg.)	Total ascorbic acid in 10 g. of dry seeds (in mg.)	Per cent. excreted total ascorbic acid (with mean deviation)
Mung ..	4	0·15	0·17	1·63	10·4±1·61
	8	0·33	0·42	3·11	13·5±0·53
	15	0·54	0·72	4·80	15·0±0·83
Masoor ..	4	0·11	0·15	1·01	15·0±1·48
	8	0·23	0·34	1·85	18·4±1·33
	15	0·51	0·69	3·20	21·6±1·35
Black pea ..	4	0·09	0·12	1·01	11·9±0·82
	8	0·21	0·27	1·87	14·4±0·54
	15	0·62	0·72	3·60	20·0±0·88

As reported earlier,[4] no dehydro-ascorbic acid was present in the seeds either after overnight soaking or during the initial phases of subsequent germination. The steady increase in its proportion with steeping would suggest increased oxidation of the excreted ascorbic acid; it is possible that there has also been some partial destruction of the oxidized form of the vitamin.

When the seeds after preliminary overnight soaking were allowed to germinate in dishes with cotton wads moistened with 300 p.p.m. solution of potassium meta-bi-sulphite, ascorbic acid could be detected in the cotton wads only during the first 24 hours (*i.e.*), on the 2nd day of germination, this period being counted from the commencement of steeping.[4] Results are given in Table II.

TABLE II

Excretion of ascorbic acid during germination

Legume	Excreted ascorbic acid in mg.	Excreted total ascorbic acid in mg.	Total ascorbic acid in 10 g. seeds in mg.	Per cent. excreted total ascorbic acid (with mean deviation)
Mung ..	0·42	0·55	8·0	6·9±1·27
Masoor ..	0·42	0·52	4·2	12·4±1·07
Black pea ..	0·50	0·66	4·7	14·0±0·81

It was ascertained that there was negligible excretion during subsequent germination over a period of 8 days.

In his experiments on amino acid excretion, Virtanen (*loc. cit.*) had observed that excretion occurred only in media capable of absorbing the excreted amino nitrogen. To find out whether ascorbic acid excretion was modified by the presence of an absorbing medium, 10 gm. lots of *mung* were soaked overnight in 300 p.p.m. solution of potassium meta-bi-sulphite in beakers and in petri-dishes lined with cotton wad (Table III). The former was merely a water medium, while the latter provided a medium capable of absorbing excreted vitamin.

TABLE III

Excretion of ascorbic acid in relation to absorbing medium

Ascorbic acid content of *mung* (10 g.) in beaker mg.	Excretion in beaker		Ascorbic acid content of *mung* (10 g.) in dish mg.	Excretion in dish with cotton wads	
	Ascorbic acid in mg.	Total ascorbic acid in mg.		Ascorbic acid in mg.	Total ascorbic acid in mg.
4·8	0·69	0·81	4·7	0·43	0·56

Excretion was higher in the beaker than in the dish by $42{\cdot}1 \pm 6{\cdot}02$ per cent. It is obvious that the low figures of free and total ascorbic acid in the dish have been due to pronounced irreversible oxidation of the vitamin on account of the larger surface area of exposure.

DISCUSSION

In the germinating seed, the leaf is the seat of maximum ascorbic acid elaboration, while the radical and stalk has a significantly low value.[4] The excretion observed had taken place when the radical had not pierced through the seed coat. This would mean that the ascorbic acid was secreted not from the radical but probably through the seed coat by osmosis. The absence of excretion at later stages of germination must be due to the fact that the ascorbic acid content of the radical which is then the only part in contact with the medium is very low so that, even if a part of the vitamin is secreted into the surrounding medium, the quantity would be very small.

Though excretion of ascorbic acid is appreciable as compared to the ascorbic acid contents of the seed on 1st day of steeping, the total excretion is negligible when compared to the ascorbic acid potency of fully germinated seed. Thus, in *mung* seeds, the ascorbic acid excreted during soaking is about 0·7 gm. while the ascorbic acid content of the seeds that cause this excretion is about 5 mgm., but the same quantity of seeds would have elaborated about 15 mgm. of ascorbic acid after germination for 5 days.

Summary

Excretion of small but definite and measurable amounts of ascorbic acid takes place during the preliminary period of soaking prior to germination of leguminous seeds. There is no secretion of the vitamin into the surrounding medium during the subsequent normal germination period.

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STUDIES ON EARLY DEVELOPMENT AND REGENERATION IN SOME INDIAN MARINE SPONGES

(With 12 Sheets of Text-Figures and 1 Plate of 12 Photographs)

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CONTENTS

PAGE

1. STUDIES ON *Callyspongia diffusa** 274
   i. Development of Asexual Larva 274
   ii. Structure and Metamorphosis of the Asexual Larva .. 282
   iii. Experimental Studies on Dissociation and Subsequent Regeneration 295
2. NOTES ON THE EARLY DEVELOPMENT OF *Tedania nigrescens* and *Hircinia* sp. 303

INTRODUCTION

WHILE the taxonomy of the Indian sponges has been the subject of many papers† little attention seems to have been devoted to the study of their soft parts and embryology. Prof. R. Gopala Aiyar, former Director of the University Zoological Laboratory, suggested to me at the instance of Dr. H. Srinivasa Rao, that the study of the life-histories of the common Madras forms would be interesting. Studies incorporated in the following pages relate to the development of asexual larva in *Callyspongia diffusa* and its metamorphosis, dissociation and subsequent regeneration in *Callyspongia diffusa* and the early development of *Tedania nigrescens* and *Hircinia* sp. The work has taken about three years for completion. Since it was carried out during the war period, visits to the Harbour and collection of specimens were prevented for sometime by the Military Authorities.

I wish to express here my deep gratitude to Prof. R. Gopala Aiyar, under whose supervision and guidance the work was carried out. I am

* A preliminary report in the form of an abstract has been published in *Proc. Ind. Sci. Congr.*, 1943, **29** (3), 148. The sponge was referred to therein as *Chalina* sp.

† A list of references is given in the article by Dr. H. S. Rao on "The Progress of Zoology in India during the Past 25 Years," reprinted from *The Progress of Science in India During the Past 25 Years*, published by the Indian Science Congress Association, 1938.

grateful to the Syndicate of the Madras University for the award of a Research Studentship. I am indebted to Dr. H. Srinivasa Rao, formerly of the Zoological Survey of India, for the identification of the sponge *Callyspongia diffusa* and for the constant encouragement given to me. I am also grateful to Dr. M. Burton of the British Museum (Natural History) for his kind criticisms and valuable suggestions.

1. STUDIES ON *CALLYSPONGIA DIFFUSA*

i. Development of Asexual Larva

Reproduction in sponges conforms to one of the following types, *viz.*, vegetative, sexual, and asexual. The first two appear to be of almost universal occurrence, while the third—asexual process—is uncommon.

In the asexual method specialised reproductive cells group themselves into congeries and form gemmules or internal buds as in fresh-water sponges which may be said to be typical in this respect. A ripe gemmule consists of a solid mass of polygonal cells heavily loaded with yolk granules, surrounded by a capsule which bears a perforation, known as the micropyle, through which, under favourable circumstances, the cellular mass inside creeps out to form a new sponge.

The occurrence of bodies similar to gemmules in fresh-water sponges has been observed in a few marine forms also (Topsent,1888; Wilson, 1894; Minchin, 1900). In *Ficulina* and *Suberites* (Sollas, 1909) gemmules form a complete layer of one to four tiers attached to the surface of support of the sponges. An opening corresponding to the micropyle of fresh-water sponges is usually present in the spongin coat. Presumably the contents of the gemmules leave the sponge by the canal system.

Larvæ produced from gemmules or internal buds have been observed in a few marine forms. Wilson (1891, 1894 and 1902) described for the first time, the development of gemmules into ciliated larvæ in *Esperella fibrexilis* and *Tedania brucei*. Ijima (1901) described the asexual larvæ in Hexactinellid sponges and traced their origin to the congeries of archæocytes attached to the walls of the chambers.

A detailed account of my observations on the gemmule and asexual ciliated larva of *Callyspongia diffusa* is given here.

Material and Methods

Callyspongia diffusa (Pl. XVII, Fig. 1) was obtained in the Madras Harbour, a regular and abundant supply of which is available throughout the year encrusted on the walls of the Harbour below low watermark. From

the encrusting base arise numerous small cylindrical minutely hispid branches bearing oscula at their extremities. Colonies of hydroids and Polyzoa are often associated with them. The colour of the living sponge is a bright violet which changes to yellowish brown on preservation in alcohol. In a mature sponge gemmules are seen as white specks, being more abundant on the attached surface.

Both Zenker's and Flemming's fluids fix the sponge well with the advantage of a slight softening of material. Sections were stained in Iron Hæmatoxylin and counterstained with Orange G or Fuchsin S, and Mann's Methyl Blue Eosin. Feulgen's stain was used to aid the study of the nuclei in the formation and development of the gemmule.

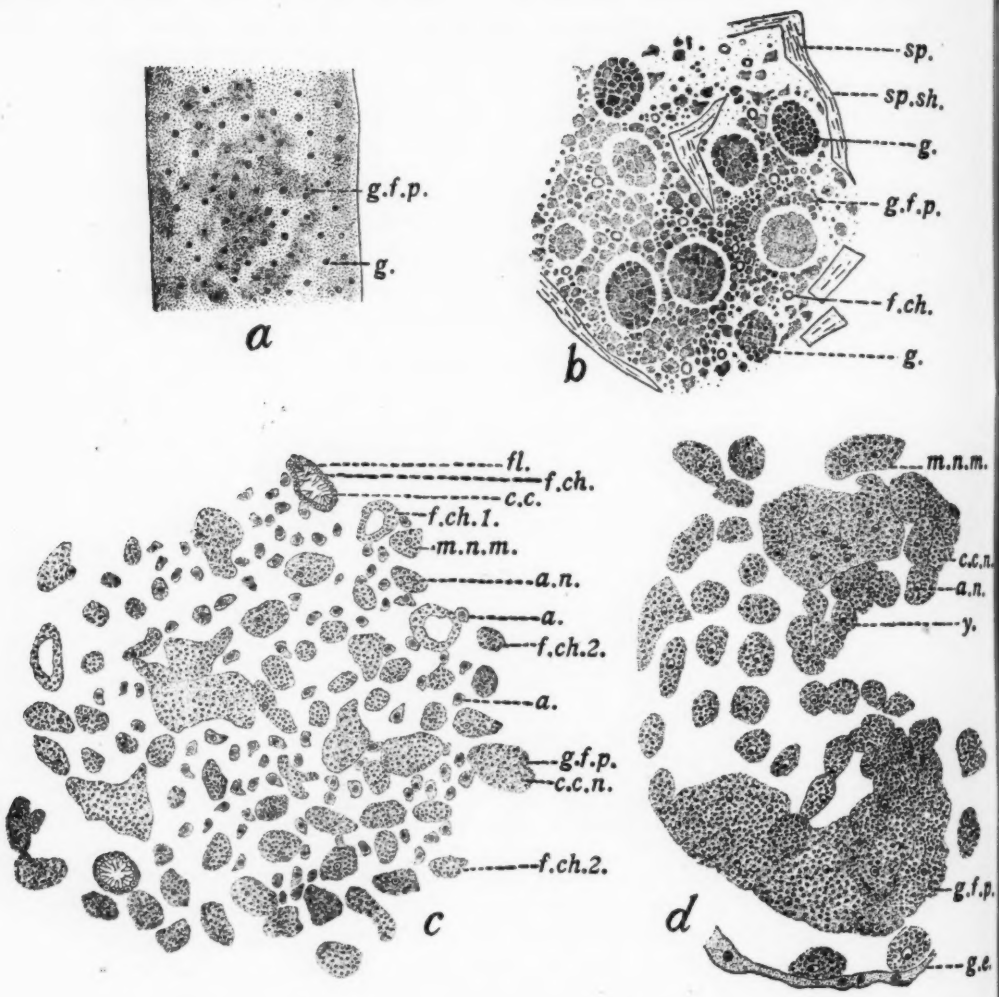
Formation of Gemmules

Kept in large vessels of fresh clean seawater in the Laboratory, the sponges liberate larvæ, immediately they are brought from the Harbour. In what may be termed mature sponges bearing gemmules seen as tiny white spots scattered all over the surface (Fig. 1 *a*; Pl. XVII, Fig. 2) the liberation of the larvæ is in much larger numbers.

In the course of the formation of the gemmules the violet colour, which is due to the presence of pigments lodged in the cells best seen in young sponges with slender cylindrical branches, is gradually lost as the pigment cells get used up in the formation of gemmules which appear as white patches. A feebler flow of water through the canal system appears to be the immediate result of the growth of these gemmules which may press upon the canal system and reduce its capacity to carry the normal volume of water. Burton's (1933) statement that in a mature sponge filled with embryos there is less free flow of water seems to corroborate my observations.

The distribution of gemmules in the body of the sponge is haphazard as may be observed in sections. In the region of the sponge without gemmules the presence of healthy flagellated chambers can be noticed with amœbocytes in between them. But in regions where gemmule production has commenced the flagellated chambers disappear while amœbocytes and congeries of multinucleate masses abound (Figs. 1 *b* and *c*; Pl. XVII, Figs. 3 and 4).

The canal system of the sponge is thrown into disorder with the growth of the gemmules. While the smaller canal systems are obliterated by the growing gemmules, the larger ones are either disrupted by the escaping larvæ or have their linings broken down.



TEXT-FIG. 1. *Callyspongia diffusa*.—(*a*) Surface view of the sponge with reproductive bodies in various stages of growth. (*b*) Transverse section of sponge through gemmular district, 10 × 8. (*c*) Portion of gemmular region, 10×40. (*d*) Section of sponge showing beginning of the formation of gemmule, 10×40.

a., amœbocyte; *a.n*., amœbocyte nucleus; *c.c*., collar cell; *c.c.n*., collar cell nucleus; *f.ch*., flagellated chamber; *f.ch*. 1, flagellated chamber with no flagellæ; *f.ch*. 2, flagellated chamber with lost flagellæ and chamber; *fl*., flagellum; *g*., gemmule; *g.f.p*., gemmule forming plasmodium; *g.e*., gemmular envelope; *m.n.m*., multinucleate mass; *sp*., spicule; *sp.sh*., spicular sheath; *y*., yolk granules.

Sections of the sponge through the region where gemmules are abundant reveal different stages of gemmule formation. All gradations from fully developed larvæ to small groups of multinucleate masses can be noticed.

During gemmulation, the flagellated chambers undergo remarkable changes. The internal cavities of the chambers are gradually reduced in size until they finally disappear. The cell boundaries of the collar cells vanish with their cytoplasm aggregated into a single continuous mass. The collars and flagellæ are also withdrawn. These changes can be studied from Fig. 1 *c*. The conversion of the flagellated chamber into a multinucleate mass is also attended with certain changes in the nuclei of the collar cells. Besides the gradual concentration of the chromatin of the collar cell nucleus it takes on a deeper and more even stain. With further concentration of the chromatin the size of the nucleus which is normally $1\frac{1}{2}$ to 2 μ, is reduced to 1 μ. In the regions of the sponge free from gemmulation, healthy flagellated chambers are met with in the peripheral regions of the sponge body where they carry on their normal activities. Between the active flagellated chambers and the multinucleate masses there are some transitional stages which may be seen in the sections of the sponge from the periphery to the base. The changes in the flagellated chambers is followed by the obliteration of the canal spaces and the rupturing of the trabeculæ.

Transverse sections of the sponge through the gemmular region also reveal clearly that the plasmodial masses described above are associated with amœbocytes, the cytoplasm of which is usually fused with that of the multinucleate mass (Fig. 1 *d*). The amœbocytes entering this union may be lying just outside the original collar cell chamber or lying adjacent to the epithelial cells of the canals. The union of the amœbocytes seems to proceed simultaneously with the transformations of the flagellated chamber.

The normal multinucleate mass formed from a single chamber undergoes further reduction in size owing to the condensation of the internal cytoplasm to admixture with very fine particles of yolky matter required during later stages of development. In cases where more than one chamber go to form the multinucleate mass, the latter resolves into daughter multinucleate masses corresponding to those derived from a single flagellated chamber. In a fully developed multinucleate mass there is a centrally placed nucleus surrounded by a large number of collar cell nuclei. The centrally placed large nucleus belongs to one of the amœbocytes that entered this plasmodial aggregation. So each multinucleate mass provided with an amœbocyte and a large number of choanocytes forms the unit of a gemmule. These masses coalesce and give rise to the gemmule. Their compact

amœboid nature helps movement through the sponge mesenchyme and aggregation to form congeries. The difference between reproduction in the initial and later stages lies in the fact that in the former multinucleate masses are small and amœboid and appear to converge to a point, and in the latter they tend to be more or less stationary constituting the focal points for other gemmule-forming cells. The union of the amœbocyte with a large number of choanocytes to form the multinucleate condition in the development of the gemmule appears to be a process not unlike that of the plasmodial aggregations of *Spongilla lacustris* described by Evans (1894).

The darkly staining cytoplasm of the multinucleate mass often obscures in sections the choanocyte nuclei. To distinguish clearly the nuclei from the darkly staining cytoplasm, Feulgen's Reaction was tried with success, in which the nuclei alone take a brilliant violet colour leaving the cytoplasm unstained (Figs. 2 *a* and *b*). The amœbocyte nucleus (3 to 4 μ) lies centrally in the multinucleate mass with the chromatin mass (1 μ) in a clear nuclear space. The choanocyte nuclei surround the centrally placed amœbocyte nucleus in the common cytoplasm.

Large numbers of the multinucleate aggregations of the kind described above come together and fuse resulting in a common mass. Sometimes small bodies so formed unite with similar ones to form a gemmule. The outer contour is distinct without any indication of an investing membrane. The follicle forming an envelope for the gemmule is built up by amœbocytes lying outside the gemmule. These cells resemble the epithelial cells in having a nucleus (without nucleoli) 2 to 3 μ and a hyaline cytoplasm. They migrate towards the gemmule and begin to invest the exterior. Fusion of such adjacently placed cells results in a continuous unicellular envelope for the gemmule.

The gemmule when fully formed is a very compact structure with the multinucleate bodies tightly packed together and their boundaries less and less distinct until they disappear and becomes a single continuous cytoplasmic mass with scattered amœbocyte nuclei and choanocyte nuclei. Unless correctly destained the nuclei in the gemmule cannot be made out but Feulgen's preparations however obviate the necessity for destaining.

Differentiation of the Gemmule into a Swimming Larva

The whole process of the differentiation of the gemmule in *Callyspongia diffusa* into the ciliated larva is a rearrangement of the cells already present unlike what has been described in *Esperella fibrexilis* (Wilson, 1894). The choanocyte nuclei become more prominent and gradually assume their original

shape and appearance and organise bits of protoplasm around them. Those that are situated in the interior begin to migrate to the outer margin of the gemmule and join the ranks of the collar cells that were originally present at the periphery (Fig. 2 *c* and Pl. XVII, Fig. 5). On reaching their destination, they change their shape. The cytoplasmic part is drawn out into a slender rod-like portion and develop cilia at the outer extremity (Figs. 2 *d* and *e*).

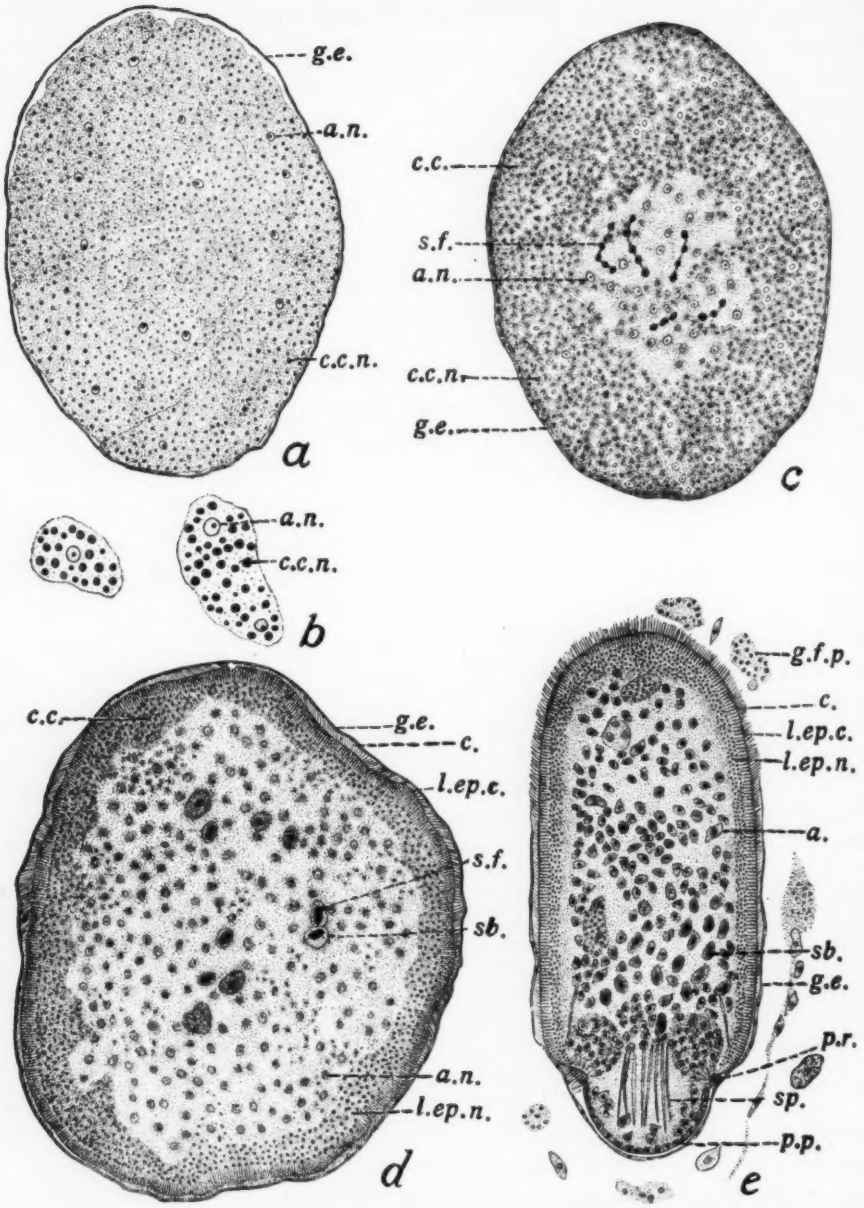
The alignment of the collar cells does not extend all through the outer margin of the gemmule as will be seen from Fig. 2 *e*. A small portion which later becomes the posterior pole of the swimming larva is left without the ciliated epithelial cells and in this region the collar cells do not come to occupy the periphery. Pigment is seen deposited in the area separating the posterior pole from the anterior region. The cilia developed along this pigment ring are longer than those of the slender ciliated epithelial cells. Pl. XVII, Fig. 6, is that of mesenchyme of the sponge wherein fully differentiated larva from gemmule can be seen.

The amœbocytes which remain in the interior give rise to the inner mass of the larva. Even at such an early stage spicules and spongin fibres make their appearance. The spicules are developed in the posterior pole of the differentiating gemmule where they group themselves into a bundle. Spongin fibres are seen formed at the centre where they are recognised by their taking deeper stain.

The shape and size of the gemmule are altered when it is transformed into a larva, the oval shape giving place to the oblong. As the stored food material in the gemmule is used up, the cytoplasm becomes gradually clearer and the tight-fitting gemmular envelope becomes very loose providing space for the movements of the larva which bring about its gradual rupture at the anterior end of the larva, due probably to the more violent ciliary action in progression at this end. On coming out of the envelope the larva is caught in the eddy of the excurrent canal system and pushed out of the osculum to the outside.

General Remarks on the Asexual Origin of the Ciliated Larva in Certain Sponges

Wilson (1894) has observed that the asexual larvæ of *Esperella fibrexilis* and *Tedania brucei* generally resemble those developed from the segmentation of the eggs in other species. He found that ' mesoderm ' cells with yolk collected together to form a rounded mass the outer cells of which formed



TEXT-FIG. 2. *Callispongia diffusa*.—(*a*) Section of gemmule (Feulgen preparation), 10×40. (*b*) Section of multinucleate mass (Feulgen preparation), 10× 90. (*c*) Section of differentiating

gemmule with the collar cells in movement to the periphery, 10×40. (*d*). Section of differentiating gemmule showing the collar cells occupying a peripheral position, 10×40. (*e*) Section of larva formed from gemmule inside the parent body, 10×40.

a., amœbocyte; *a.n.*, amœbocyte nucleus; *c.*, cilium; *c.c.*, collar cell; *c.c.n.* collar cell nucleus; *g.f.p.*, gemmule forming plasmodium; *g.e.*, gemmular envelope; *l.ep.c.*, larval ciliated epithelial cell; *l.ep.n.*, nucleus of the larval ciliated epithelial cell; *p.p.*, posterior pole; *p.r.*, pigment ring; *sb.*, spongoblast; *s.f.*, spongin fibre; *sp.*, spicules.

the follicle. The growth of the gemmule was by cell division and by the fusion of the smaller gemmules resulting in a mass of closely packed cells full of fine yolk. The mesoderm cells involved in this gemmulation are just like any other amœbocytes in the mesenchyme except that they are packed with finely granular yolk in consequence of which they stain well.

Serious doubts have been entertained by Maas (1893 and 1896) and Minchin (1897) on the accuracy of Wilson's observations concerning the asexual larva. *Callyspongia diffusa* reproduces itself throughout the year by gemmule formation. Each unit of the gemmule is a multinucleate aggregation of a single amœbocyte and numerous collar cells, which is amœboid and moves to a common point along with others to form the gemmule. The behaviour of these multinucleate masses leaves one in no doubt about the asexual origin of the ciliated larva.

In *Esperella fibrexilis* the larvæ are formed from amœbocytes laden with yolk collecting together secreting a gemmular sheath around them. The growth of the gemmule is due either to the multiplication of cells already present inside the gemmule or to the fusion with other smaller gemmule. The larva is formed by the breaking up of the constituent matter of the gemmule into irregular masses of cells, which move to their respective positions in the body of the larva. In *Callyspongia diffusa* the gemmule is formed by an association of large number of multinucleate masses formed from amœbocytes and collar cells. The growth of the gemmule is achieved by the fusion of such amœboid masses. The unicellular gemmular envelope is formed outside the gemmule from amœbocytes of the mesenchyme of the sponge resembling epithelial cells and coming together around the gemmule to form a unicellular investment. The ciliated larval epithelial cells are formed from the collar cells of the sponge by a process of emigration during the differentiation of the gemmule. Ijima's (1901) account of the formation of the Hexactinellid larva agrees with that of *Esperella fibrexilis* in its asexual origin from groups of cells. An uninterrupted series of stages leading from the archæocyte congeries attached to the surface of the flagellated chambers led him to conclude that the larvæ were formed from the archæocyte groups. The growth of the congeries takes place not only by

cell division but by fusion of originally separate groups. The archæocytes concerned in the formation of these asexual bodies are small spherical or ovoid bodies measuring 2 to 4 μ in diameter. In *Callyspongia diffusa* the range in size of the multinucleate bodies which form the gemmule is 10 to 12 μ and the bodies are much bigger than the amœbocytes of the mesenchyme.

The unique feature in the gemmule development of *Callyspongia diffusa* is the participation of the collar cells in the formation of the multinucleate aggregations. The researches of Dendy (1915), Gatenby (1920, 1927) and Duboscq and Tuzet (1937) prove clearly that collar cells also take part in the sexual reproduction of the sponges they describe by transforming into reproductive elements.

In conclusion it may also be stated that the development of the asexual larvæ from gemmules in *Callyspongia diffusa* is a possible adaptation to the necessity for survival and rapid dispersal in the intertidal zone.

SUMMARY

1. The development of the asexual larva in *Callyspongia diffusa* is described for the first time.

2. *Callyspongia diffusa* reproduces asexually by means of gemmules which give rise to larvæ.

3. A gemmule is formed by the congregation of a large number of multinucleate aggregations arising from the amœbocytes and the collar cells of the sponge.

4. In the transformation of the gemmule into the ciliated larva the collar cells which contribute to the formation of the gemmule assume a position external to the amœbocytes to give rise to the ciliated larval epithelial cells.

5. The origin of a ciliated sponge larva from a single cell, the egg, or a group of cells is discussed.

ii. Structure and Metamorphosis of the Asexual Larva

According to Minchin (1900, p. 67) 'there is scarcely any zoological problem which would appear, from a study of the literature alone, to be so confused and difficult as the embryonic development of sponges. The difficulty proves, however, to be due not so much to the nature of the objects themselves as to the many prejudices and preconceived notions with which they have been studied'. In taking up the studies of the kind briefly dealt

with in the following pages the writer's object has been to describe as accurately as possible the structure and metamorphosis of the asexual larva of *Callyspongia diffusa* and add to the *by no means extensive* knowledge of the early development of Indian Sponges.

We owe our knowledge of the structure and metamorphosis of the larva of sponges mainly to the elaborate researches of Ganin (1878), Goette (1886), Maas (1890, 1892, 1893, 1896), Yves Delage (1890, 1891, 1892, 1893, 1899), Noldoke (1894), Evans (1899), Minchin (1896, 1900), Ijima (1901), Wilson (1891, 1894, 1932, 1934, 1935), Okada (1928), Meewis (1937) and Brien and Meewis (1940). The present study of the structure and metamorphosis of the larva of *Callyspongia diffusa* confirms the general theory of ' the inversion of germ layers ' so characteristic of most sponges, in bringing out clearly the fact that the larval ciliated epithelial cells give rise to the flagellated chambers of the developing sponge.

Technique

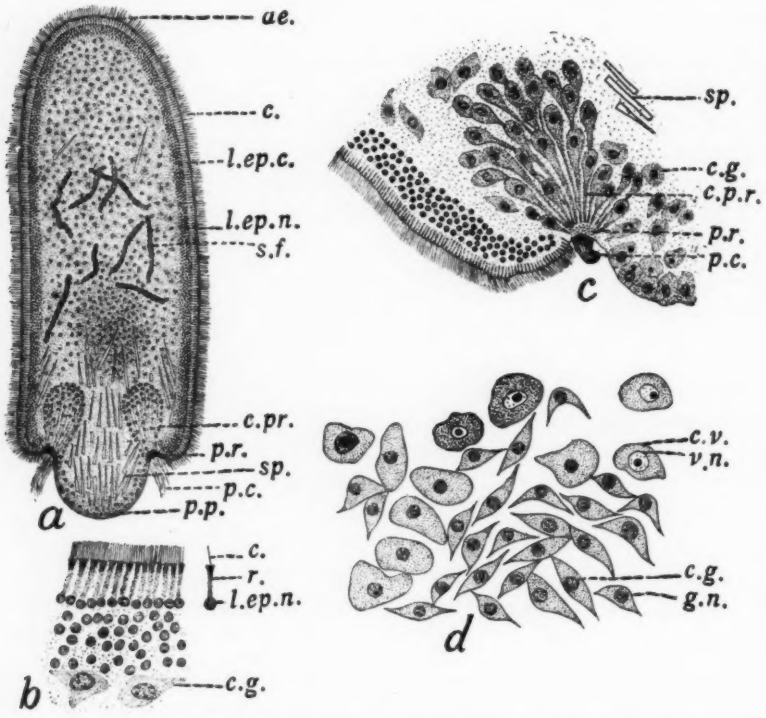
Sponges collected in the Madras Harbour were immediately transferred to fresh sea-water in large vessels. The larvæ extruded along with the streams of exhalant water issuing from the oscula, were then picked out and fixed in Bouin's, Zenker's and Fleming's fluids which gave good results. They were sectioned after embedding in paraffin and celloidin and paraffin. Of the large number of stains used, Iron Hæmatoxylin, Delafield's and Ehrlich's Hæmatoxylin, Blue de lyon, Mann's Methyl Blue Eosin, Borax Carmine and Feulgen's stain yielded good results. Whole mounts of the larvæ were made in Borax Carmine.

In the Laboratory aquaria larvæ were allowed to fix themselves to the surface of paraffin-coated watch glasses and later transferred to a larger vessel in the aquarium with a continuous supply of clean sea-water. Pieces of paraffin to which larvæ were attached were cut out with a needle and floated to the surface, and then placed in the preservative. Sections of seaweeds on which larvæ had settled were also useful to study the stages of metamorphosis.

Description of the Larva

The larva is an amphiblastula in which histogenesis has commenced (Fig. 3 *a* and Pl. XVII, Fig. 7). As soon as it is discharged from the sponge the ellipsoidal larva swims actively for about three hours executing spiral movements but avoiding light. This is followed by a bottom living phase when the larva moves briskly on the bottom of the aquarium. The larva has its posterior end rounded and the anterior pointed, and in the uncontracted

state 340 μ long as compared to 1 mm. (or 1,000 μ) of the larva of the allied *Mycale* (Maas, 1892) and 650 μ of the larva of the same sponge (Wilson, 1895). The larva suffers contraction when transferred to the fixative.



TEXT-FIG. 3. *Callyspongia diffusa*.—(*a*) Longitudinal section of the larva, 10×40. (*b*) Part of section of the larva through the larval epithelial cells, 10×90. (*c*) Section of the larva passing through the pigment ring, 20×40. (*d*) Section of the larva showing the cells of the inner mass, 10×90.

ae., anterior pole; *c.*, cilium; *c.g.*, cells with granular nucleus; *c.p.r.*, cells of the pigment ring; *c.v.*, cells with vesicular nucleus; *g.n.*, granular nucleus; *l.ep.c.*, larval ciliated epithelial cells; *l.ep.n.*, nucleus of the larval ciliated epithelial cells; *p.c.*, cells of the pigment ring; *p.p.*, posterior pole; *p.r.*, pigment ring; *r.*, rod-like portion of the cytoplasm of the epithelial cells; *s.f.*, spongin fibre; *sp.*, spicule; *v.n.*, vesicular nucleus.

In describing the histology of the larva the terms ' ectoderm ', ' endoderm' and ' mesoderm ' have been purposely dropped to avoid confusion. An outer ciliated epithelium except over a small area at the posterior end and an inner mass of cells can be distinguished. The epithelial cell is flask-shaped (Fig. 3 *b*) the cytoplasmic neck portion bearing the cilia being rod-

shaped and the nuclear body portion being rounded. As the distal ends of the rod-shaped portions are funnel-shaped the rods do not appear to be continuous. As the epithelial nuclei are closely pressed together they appear to be disposed in three or four separate strata. The difference in the shape of the nuclei which are spherical or oval is due to the nature of the section, tangential or radial. The fused outer ends of the cells form a marginal membrane in which cell outlines are indistinguishable, exhibiting a denser and more opaque consistence in this portion than in the thinner waist-like constriction. The flagellum 6 μ to 7 μ is as long as the cell body. Between the anterior ciliated region of the larva and the posterior nonciliated portion there is a distinct pigment ring of a brown colour in which the cilia are prominent and long.

There are four kinds of cells in the body of the larva, *e.g.*, (1) the amœbocytes with granular nuclei (referred to hereafter as g.n. cells), (2) cells with vesicular nuclei (referred to hereafter as the v.n. cells), (3) sclerobIasts or the spicule-forming cells, and (4) the spongoblasts or the spongin-forming cells. The g.n. cells (Fig. 3 *c*) are usually found more towards the anterior end of the larva. With the formation of the larva from the gemmule, the cells in the anterior region gradually transform themselves into g.n. cells. The nucleolus inside the nucleus breaks into a large number of small granules placed at the nodes of a fine reticulum. The cytoplasm becomes clearer and assumes an irregular form which does not admit of proper measurement. The g.n. cells, 7 μ in diameter on the flattened side, which found as a complete layer under the larval ciliated epithelial cells, also line the exposed posterior pole of the larva. They appear to be a class of cells, capable of giving rise to a flat epithelium when situated near the surface or, as it happens later in metamorphosis, to a lining if they are inside the metamorphosing sponge. They bear great similarity to the epidermal cells of Delage. The v.n. cells (4 μ to 7 μ in diameter), are highly plastic and correspond to the archæocytes which give rise to any kind of sponge tissue. They occupy the central and posterior portion of the inner mass. The nuclei of these cells are the largest in the larva (4 μ in diameter), their size depending on the state of larval development. The centre of the nucleus which has a very thin nuclear membrane is occupied by a nucleolus or the central corpuscle. Between the nucleolus and the nuclear membrane is a fine reticulum at the nodes of which chromatin granules are located. The size of the nucleolus is dependent upon the number and size of the chromatin granules placed at the nodes of the reticulum. When the nucleolus is big, the chromatin granules around are few and far between. In a few cells it

has been observed that a big nucleolus is situated at the central portion of the nucleus, leaving a clear space around it, free from nuclear reticulum and chromatin granules. The thick cytoplasm is finely granulated. Evans (1899) describes the presence of nutritive vacuoles, yolk bodies, and refringent granules inside the v.n. cells observed by him. No such inclusions have been found in similar cells in the larva of *Callyspongia diffusa*. It will be seen from the above description that these cells resemble the amœbocytes present in the differentiating gemmule. In fact they are continued from the gemmule into the larva as the archæocytes destined to give rise to any kind of tissue. The scleroblasts and spongoblasts are formed from these cells.

The pigment ring already referred to is formed from the secretions of special types of cells derived from the inner mass (Fig. 3 *d*). During the differentiation of the inner mass in the developing larva, the v.n. cells, in the region separating the anterior and posterior end, gradually assume a peculiar shape and function. Their nuclei become granular, the chromatin gets deposited in the nuclear reticulum, becoming oval in shape and 3 μ in diameter. The cytoplasmic portions of the cells are drawn out towards a common point where cell boundaries vanish. The cells develop flagellæ 10 μ in length. In a section of the larva, the cells concerned in the formation of the pigment present a flask-shaped appearance. In a swimming larva the long and prominent cilia of this pigment ring curve and invest the posterior pole of the larva seeming to form a sort of cage for it.

There appears to be no space or cavity inside the inner mass, the interior being fully packed with cells as shown in sections of the larva. Wilson (1935) however, describes collenchymatous spaces inside the larva of *Mycale*.

Development of Fibrils and Spicules

In sections of the entire larva well-developed fibrils and spicules may be seen (Fig. 3 *a*), the former in the centre of the larva and the latter confined to the posterior region. The elastic fibrillæ are formed as follows:— A large number of v.n. cells co-operate in the formation of a single fibril. The cells in question develop in their cytoplasm minute dark granules (Fig. 6 *c*). The spongin makes its appearance by the side of the nucleus in a cell as a spherule which takes an even and deep stain. With Mann's Methyl blue eosin the spherules are stained bright red. The spherule inside the cell grows to assume the shape of a rod. Adjacent cells arrange themselves in a row in the form of a string giving a beaded appearance (Fig. 6 *d*). The rods of spongin get elongated with the result that a long slender fibril, without a trace of their previous jointed condition, is enclosed in a sheath

of protoplasm. With greater growth the elastic fibrillæ come to lie in the inner mass, losing their enclosing cells, the nuclei of which seem to be scattered amidst the cells of the inner mass.

Spicules of the monaxon type make their appearance very early in the larvæ, together with the fibrils described above. They make their first appearance in the v.n. cells, mostly in the posterior region of the larva. In the swimming larva they are arranged in the form of a bundle with a converging point at the posterior pole (Fig. 3 *a*). Two stages in the growth of the spicule are shown in Fig. 6 *e*. To begin with the spicule is enclosed in a scleroblast as an intracellular secretion. The cytoplasm of the scleroblast remains very clear and without granules. The nucleus of the cell loses its nucleolus and becomes granular. As the spicule increases in size it outgrows the scleroblast assuming a fusiform shape. With the further growth of the spicule the scleroblast adheres to the shaft of the spicule and finally disappears when the spicule attains full size. Microscleres are not developed in the larva or in the adult.

Motile Phase of the Larva

The motile phase of the *Callyspongia diffusa* larva covers a period of about six to eight hours after discharge from the parent as compared to the 24 to 72 hours of *Mycale* as observed by Wilson (1895). This shortening of the duration of the motile phase in *Callyspongia diffusa* is probably a phenomenon common among invertebrates whose development is accelerated in tropical waters. The absence of nutritive vacuoles or yolk granules in the cytoplasm may also have contributed to the abbreviation of the larval period. Where the substratum is unsuitable for fixation the larvæ seem to sink down to the bottom but remain motile. If the bottom of the culture vessel is strewn with flat weeds (*ulva*), the larvæ attach themselves in about three hours' time to the under-surface of the weeds away from light. On a smooth surface as on a paraffin-coated bottom they take about seven hours to attach themselves. On glass surface, however, they take eight hours.

Reproductive Period in the Sponge

Sponge larvæ are liberated throughout the year, but more profusely during the rainy season, especially between September and November. This is in contrast to the definite breeding seasons of sponges in temperate climates (*cf.*, Orton, 1920). The larvæ from the deeper parts of the sponge escape through the osculum with the excurrent stream of water, but those from the peripheral regions of the sponge body seem to escape through

minute holes in the skin which is apparently broken through by the escaping larvæ. It is not certain if death occurs in *Callyspongia diffusa* after reproduction, but judging from the pluripotency of the archæocytes of the sponge, it is quite possible that the sponge gets regenerated after the reproductive period.

Metamorphosis

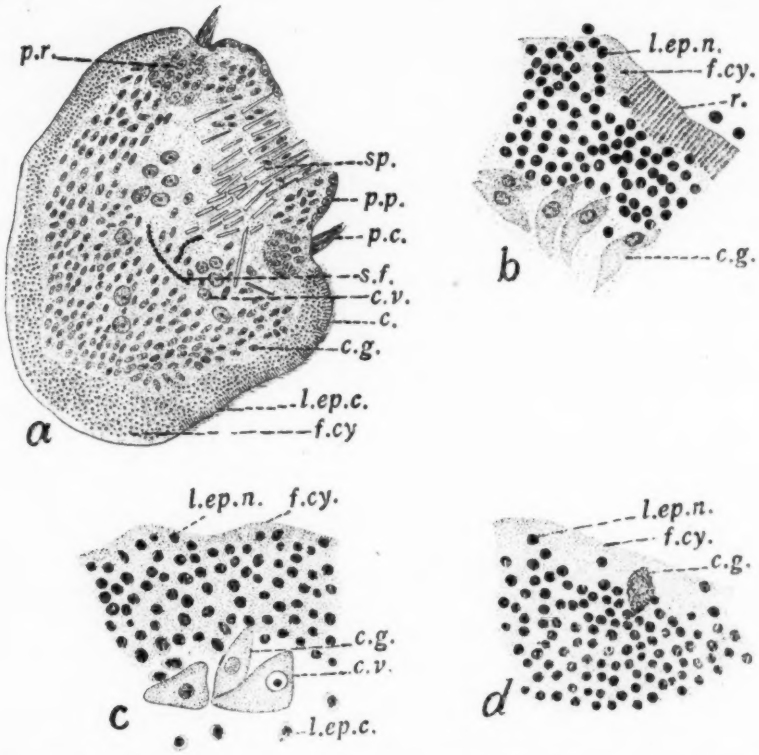
The surface moving phase of the larva is followed by a bottom living phase, during which the larva creeps along the periphery of the bottom of the vessel. Before fixation, the larva executes a rotatory motion on its posterior pole as pivot, as in a spinning top. This motion may be an attempt to select a suitable substratum for fixation. The larva, however, attaches itself to the substratum by the anterior pole or sometime by the side not farther away from the anterior pole.

The first indication of metamorphosis in the late motile larva is seen at the anterior end, where the ciliated epithelial cells show signs of inactivity with their nuclear stratum disorganised. The rod structure of the epithelial layer in this region disappears giving rise to a confused mass of cells. But at this stage the slow motility of the larva is maintained by the flagellated cells of the other regions (Fig. 4 *a*).

The motile phase of the larva is followed by the 'pupal' phase. The term 'pupa' is used by Minchin (1900) to indicate the stage in development of the sponge between the actual fixation of the larva and the formation of the dermal pores and osculum of the metamorphosed sponge. The first result of fixation is the flattening of the larva in an antero-posterior axis. The posterior pole of the larva along with the pigment ring and the spicules remain exposed to the surface (Pl. XVII, Fig. 8).

During the pupal period a series of migrations of the cells takes place which ultimately brings about the metamorphosis of the sponge. It is observed that large numbers of ciliated epithelial cells at the anterior end, which by now have lost their cilia and cell boundaries, gradually find their way to the interior. Simultaneous with the passing in of these cells to the interior, the cells of the inner mass, more especially the g.n. cells, insinuate their way to the exterior and come to occupy the position of larval ciliated epithelial cells. At this stage all the ciliated cells which once occupied the anterior end are well within the interior of the pupa, while in the remaining portions the ciliated cells remain in a more or less compact layer.

The migration of the g.n. cells is not of uniform character as there is considerable variety in their mode of migration. The number of points



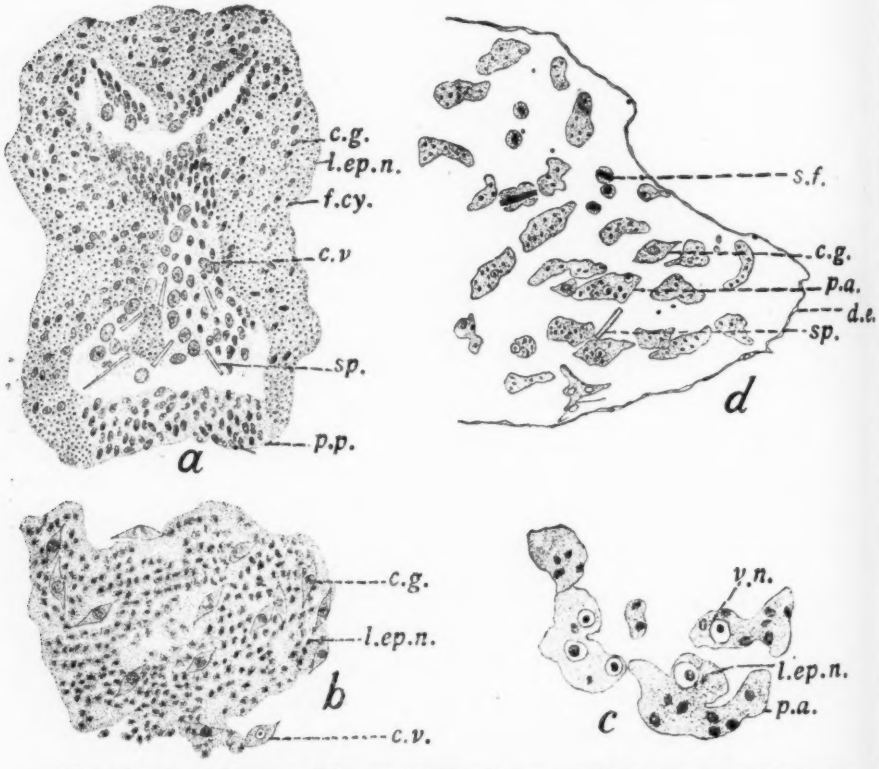
TEXT-FIG. 4. *Callyspongia diffusa.*—(*a*) Longitudinal section of larva beginning to metamorphose, 10×40. (*b*) Section of attaching larva passing through the larval epithelial region, 20×90. (*c*) Section of larva passing through the larval epithelial region showing the dissolution of the rod structure, 20×90. (*d*) Section of the attaching larva passing through the larval epithelial region showing the emigration of one g.n. cell to the periphery, 20×90.

c., cilium; *c.g.*, cells with granular nucleus; *c.v.*, cells with vesicular nucleus; *f.cy.*, fused cytoplasm of the larval epithelial cells; *l.ep.c.*, larval ciliated epithelial cells; *l.ep.n.*, larval epithelial cell nucleus; *pc.*, cilia of the pigment ring; *p.p.* posterior pole; *p.r.*, pigment ring; *r.*, rod structure of the larval epithelial cells; *s.f.*, spongin fibre; *sp.*, spicule.

through which the cells find their way out may be few or many. If the cells migrate through many points the flattening of the larva is quick. Slow migration on their part leads to the retention of the ciliated cells on the surface for a longer time.

The process of emigration of the g.n. cells and the immigration of the ciliated cells can be studied from Figs. 5 *a* and *b*. In this stage all the larval ciliated cells, which have lost their cilia and cell boundaries, are on their way

to the interior of the pupa. The rod-like structure of their cytoplasm is no longer present. The orderly cohesion of these cells forming a stratum which was seen in the larva is absent but instead they are seen to be thrown into confusion (Figs. 4 *b*, *c* and *d*). Fig. 4 *b* illustrates the stage of the beginning of disorder in the ciliated epithelial cells. The rod structure in one portion has disappeared while it is retained intact in certain other places. The stratified appearance of the nuclei is lost. The g.n. cells lie in the interior awaiting migration to the periphery. Fig. 4 *c* shows still more advance where the rod structure of the ciliated epithelial cells is completely lost in all places and their nuclei lie scattered, while the g.n. cells have begun migration to the exterior. Fig. 4 *d* is illustrative of the stage where the larval epithelial cells are moving to the interior and one of the cells with granular nuclei has come to occupy an external position.



TEXT-TIG. 5. *Callyspongia diffusa*.—(*a*) Section of an attached larva two hours after fixation 10×40. (*b*) Region of the larval epithelial cells in an attached larva two hours after fixation,

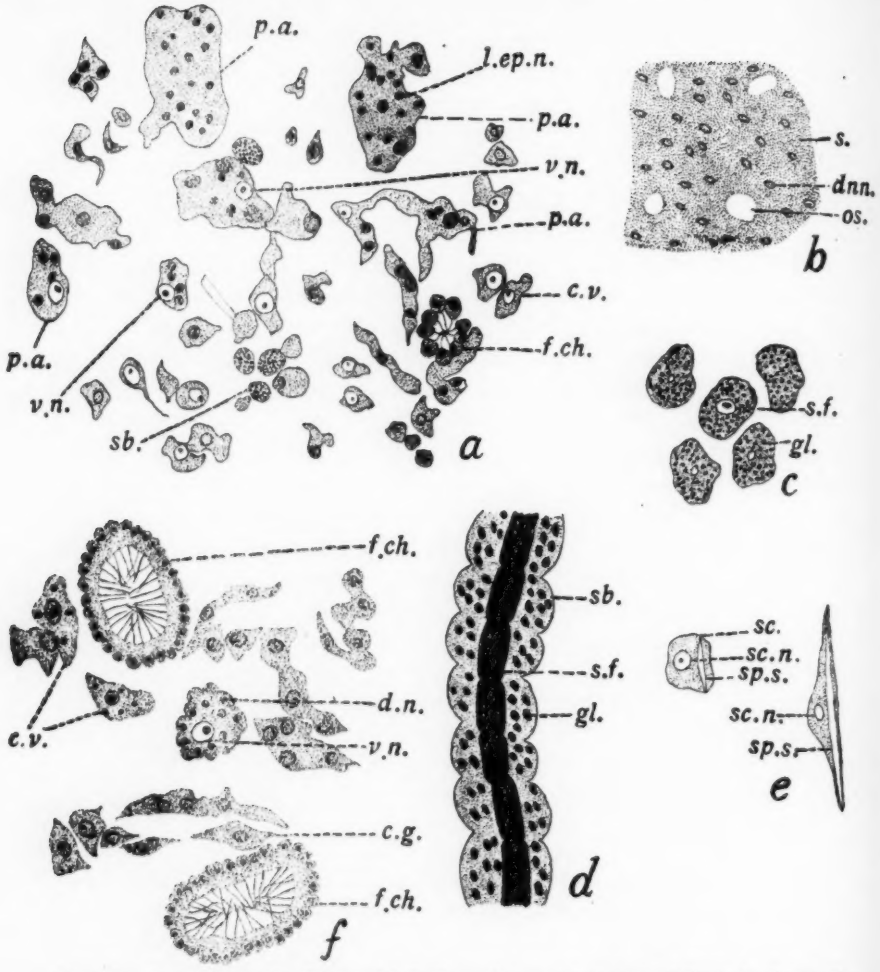
10×90. (*c*) Portion of a section of pupa 16 hours after fixation, 10×40. (*d*) Section of pupa of 16 hours' growth showing plasmodial aggregation of larval epithelial cells and v.n. cells, 20×90.

c.g., cells with granular nucleus; *c.v.*, cells with vesicular nucleus; *d.e.*, dermal epithelium, *f.cy.*, fused cytoplasm of the larval epithelial cells; *lep.n.*, nucleus of the larval epithelial cells; *p.a.*, plasmodial aggregation of larval epithelial cells and v.n. cells; *pp.*, posterior pole of the attached larva; *s.f.*, spongin fibre; *sp.*, spicule; *V.N.*, vesicular nucleus.

During metamorphosis, the cytoplasm of the larval ciliated epithelial cells shows no distinct cell boundaries. But with the progress of the process of immigration a round halo of cytoplasm appears around the nuclei. The nuclei become slightly elongated and contracted. They stain deeper than before. It is usual to find the epithelial cells in small groups. But single cells lying here and there in the interior enable us to infer that the larval epithelial cells retain their cell individuality. On closer observation it is found that the groups of larval epithelial cells are in proximity to a v.n. cell (Fig. 5 *c*). In sections of pupa sixteen hours after attachment, plasmodial aggregations of v.n. cells and groups of larval epithelial cells are visible (Fig. 5 *d*). From sections of pupa of three days' growth (Fig. 6 *a*) it is observed that on reaching the interior the larval epithelial cell groups arrange themselves in the shape of a ring leaving a space in the middle. The cells develop collar and flagella and become the typical collar cells of the adult. The space left in the middle becomes the chamber. Meanwhile the amœbocyte associated with the cell groups retires from the plasmodial aggregation. The activity of v.n. cell in this instance suggests that it helps the inward movement of the larval epithelial cell groups. The way in which the plasmodial aggregations of v.n. cell and larval epithelial cell groups behave reminds one of the processes observed in the formation of the multinucleate masses in the gemmulation of the sponge.

The g.n. cells migrating to the exterior assume a fusiform shape (Fig. 5*b*). On reaching the surface of the pupa they flatten out and meet by their edges, forming a complete and uninterrupted investment. Owing to the flattening of the cells, the nuclei become oval in shape and the cytoplasmic structure is alveolar. Ostia are developed in the epithelium. Whole mounts and tangential sections reveal that the epithelium is a symplast as described by Wilson (1910), Penny (1931) and Wilson and Penny (1930) and not a typical epithelium as observed by Tuzet (1932) (Fig. 6 *b*).

The cells with granular nuclei not only give rise to the epithelium of the new sponge but also form the marginal membrane, just beneath the epithelium. The marginal membrane may be laid down in one or two super-



TEXT-FIG. 6. *Callyspongia diffusa*.—(*a*) Section of a young sponge of three days' growth, 10×90. (*b*) Tangential section of the epithelium of the young sponge of three days' growth, 10×90; *c*., Spongoblasts in the larva formed in the inner mass, 10×90; (*d*) Fully formed spongin fibre inside the larva, 20×90; (*e*) Two stages in the formation of the monaxon spicule in the larva, 10×90. (*f*) Section of growing sponge showing the formation of flagellated chambers from the v.n. cells, 10×90.

c.g., cell with granular nucleus; *c.v*., cell with vesicular nucleus; *d.n*., daughter nucleus formed from the vesicular nucleus; *dnn*., nucleus of the dermal epithelium; *gl*., granule; *os*., ostium; *p.a*., plasmodial aggregation; *f.ch*., flagellated chamber; *s*., spymplast of the epithelium; *sc*., scleroblast; *sc.n*., nucleus of the scleroblast; *sp.s*., spicule as intracellular secretion; *vn*., vesicular nucleus of the v.n. cells; *sb*., spongoblast; *l.ep.n*., larval epithelial nucleus.

imposed layers of cells. The thickness and compactness of the marginal membrane vary at different places beneath the epithelium.

Formation of the Canal System

To begin with small lacunar spaces appear inside the sponge at the stage when the collar cells have not yet been emancipated from the plasmodial aggregations.

At first, these spaces do not have a proper flattened epithelial lining, but with the development of flagellated chambers distinct epithelial linings are formed. Uniting with similar adjacent spaces they give rise to the inhalant and exhalant canal systems of the sponge. Small subdermal cavities are formed just below the epithelium of the new sponge and communicate with the exterior by the ostia developed in the epithelium, and internally with the flagellated chambers through the inhalant canal system and the prosopyle. In the developing sponge the subdermal cavities are developed on the sloping sides while the osculum and the excurrent canals are developed in the central region of the sponge.

Development of New Flagellated Chambers

Flagellated chambers are formed either by the immigrated larval ciliated epithelial cells or by the v.n. cells. In the latter process the vesicular nucleus undergoes amitosis, with the chromatin thrown out into the cytoplasm as tiny rounded lumps of the size of the nuclear corpuscle. At one stage the cytoplasm contains a large number of small nuclei. These daughter nuclei gather around them bits of cytoplasm giving rise to the collar cells. The cavity formed in the interior of the cell becomes the chamber of the collar cells (Fig. 6*f*).

Conclusions

In the ontogeny of the silicious sponges the larval epithelial cells migrate to the interior to develop into the choanocytes of the metamorphosed sponge. A similar development occurs in certain horny and calcareous sponges. According to Ganin (1879) the flagellated cells of the larva are transformed into the epithelium of the adult, while Gotte (1886) thought that the flagellated cells of the larva are cast off entirely, the new adult sponge developing the epithelium from the cells of the inner mass. The careful investigations of Delage (1890, 1891, 1892, 1893, 1899) showed beyond doubt that the ciliated epithelial cells of the larva become the collar cells of the sponge. Evans (1899) and Wilson (1935) came to the same conclusion. Maas (1890, 1892 1893, 1896) found that the collar cells of the sponge he studied were derived

from the larval epithelial cells, but in *Oscarella* he (1893) found no reversal of cell layers, the majority of the larval epithelial cells at the surface retaining their flagella. This fact has been correlated with the ciliated condition of the dermal epithelium of the adult. In the allied *Halisarca* Meewis (1939) found that the flagellated chambers of the sponge were differentiated from the cells of the inner mass of the larva. The present studies on the development of *Callyspongia diffusa* show that the immigrated larval ciliated epithelial cells form the flagellated chambers of the metamorphosed sponge.

The method of immigration of the larval epithelial cells is described variously by different authors. According to Delage (1892) the flagellated cells of the larva are engulfed by a process of phagocytosis by the cells of the inner mass, which carry them inwards and eject them in the interior to form the flagellated chambers. Some of these cells, however, are according to him entirely digested and absorbed during the phagocytosis. According to Maas (1893) the flagellated cells of the larva immigrate on their own accord without undergoing phagocytosis, by the cells of the inner mass. Noldeke (1895) observed epithelial cells entirely destroyed in the above process, the flagellated chambers being formed by the cells of the inner mass. According to Evans (1899) the larval epithelial cells form plasmodial aggregations with the amœbocytes of the inner mass the function of which is to act as an escort to the interior where the larval epithelial cells are free to reassemble and form the flagellated chambers. Wilson (1935) explained that the movement was not of the larval epithelial cells but of their nuclei, which lie in the fused cytoplasm of all the cells of the pupa, until flagellated chambers are formed from it. In *Callyspongia diffusa* the larval epithelial cells migrate inwards assisted by the amœbocytes of the inner mass as in *Spongilla lacustris* (Evans, 1899). All the cells in the larva do not fuse to form a syncytium as observed by Wilson (1935) but it is often found difficult to distinguish between the outlines of groups of larval epithelial cells and the associated amœbocytes as is evident in my microscopic preparations.

In *Callyspongia diffusa*, the cells with vesicular nucleus play a very important role in the histogenesis of the larva and the new sponge. Some of them differentiate into cells with granular nuclei, which in turn give rise to the dermal and canalar epithelium. The scleroblasts and spongoblasts which give rise to the skeleton of the sponge are formed from v.n. cells. The flagellated chambers of the new sponge are recruited from the v.n. cells also. In short, the v.n. cells play the role of the archæocytes of the sponge.

SUMMARY

1. The larva is an amphiblastula in which precocious histogenesis is observed.

2. The flagellated cells of the larva migrate inwards during metamorphosis, usually in groups. They are seen plastered on to the walls of the amœbocytes with vesicular nuclei, which assist them in their inward movement.

3. The flagellated cells of the larva, being situated in the interior, give rise to the flagellated chambers of the metamorphosed sponge.

4. The cells with granular nuclei find their way out from the inner mass by a process of diapidesis and form the epithelium of the new sponge.

5. The cells of the vesicular nuclei are pluripotent giving rise to all the different types of cells present in the sponge.

iii. Experimental Studies on Dissociation and Subsequent Regeneration

At the suggestion of Prof. R. Gopala Iyer, the then University Professor of Zoology, Madras, I took up the study of the regeneration of *Callyspongia diffusa* from dissociated tissue cells on the lines of Wilson (1911) and Huxley (1911) in the hope of being able to throw some fresh light on this already well-known phenomenon.

Wilson (1907) discovered that when a marine sponge is broken up into its constituent cells these latter will recombine to form restitution masses having the power of transforming themselves into perfect new sponges. Muller (1911) obtained functioning sponges from the union of dissociated cells of fresh-water sponges. Huxley (1911) carried out similar experiments on calcareous (*Sycon*) and siliceous (*Reneira*) sponges. In *Sycon* he obtained shortlived functioning sponges while in *Reneira* he got spheroid restitution masses which failed to settle down and grow. It is also well known (Wilson, 1911) that sponges reformed from dissociated cells can also produce reproductive bodies. Experiments of a similar nature were conducted with hydroids by Wilson (1911), De Morgan and Drew (1913), Hargitt (1915) and Okada (1927). Galtsoff (1925) carried out investigations on the dissociated cells of *Microciona* under different conditions, while Wilson and Penny (1930) described the complete histology of *Microciona* and its regeneration after dissociation. De Laubenfels (1934) pursued similar studies on *Iotrochota birotulata* obtaining functional sponges from the restitution bodies.

Brien (1937) successfully obtained regenerated sponges from dissociated cells of fresh-water sponges.

The Reaction of the Sponge Cells to Abnormal Environment

Allied to regeneration from dissociated cells in sponges is a phenomenon known as 'reduction' described by Eugene Schultz (1904, 1906 and 1907). 'Reduction' is an inverse process in the life history of organisms in general involving gradual simplification of tissues, in the course of which complex structures acquired during ontogeny are given up in a retrogressive order. The behaviour of the organism during 'reduction' is looked upon as an adaptive response in which the needs of the organism determine what structures should be eliminated. In sponges as in other groups like Turbellaria, Hydrozoa, and Tunicata, instances of reduction occur. Lieberkuhn (1856) described how choanocytes of certain fresh-water sponges lost their distinctive form and shape with the approach of winter and assumed the character of mesenchymal cells and how the flagellated chambers were reformed with the return of favourable conditions. Metschnikoff (1879) found that the collar cells of certain marine sponges gradually changed into amœboid cells in foul water. With the return of suitable conditions the flagellated chambers reappeared. There does not appear to be any ground for the belief that the cells which formed the flagellated chamber were transformed from choanocytes. Reduction phenomena have been studied in fresh-water sponges by Muller (1911), Penny (1933) and Brien (1937) and in marine sponges by Maas (1907, 1910) and Wilson (1907). The question as to whether or not the dissociated cell elements return to the primordial formative or embryonic condition, comparable to the simplification of structure during the phenomenon of reduction is answered variously by different authors. An attempt has been made here to describe in *Callyspongia diffusa* the nature and role of the dissociated elements in the formation of the restitution bodies.

Technique used for the Experiments

Methods for growing sponges from dissociated cells have been described by Wilson (1907, 1937), Huxley (1911), Muller (1911), Galtsoff (1925), Faure Fremiet (1925, 1932), De Laubenfels (1932), Wilson and Penny (1930) and Brien (1937). The method adopted in my experiments with *Callyspongia diffusa* is the one recommended by Wilson (1937). Pieces of fine muslin cloth of 75 to 90 meshes to the square inch were used. Special care was taken to see that the sponges were clean, fresh, and tender. They were cut up into small bits about a quarter of a centimetre long, and laid in a heap in the middle of a square of muslin cloth which was folded over so as

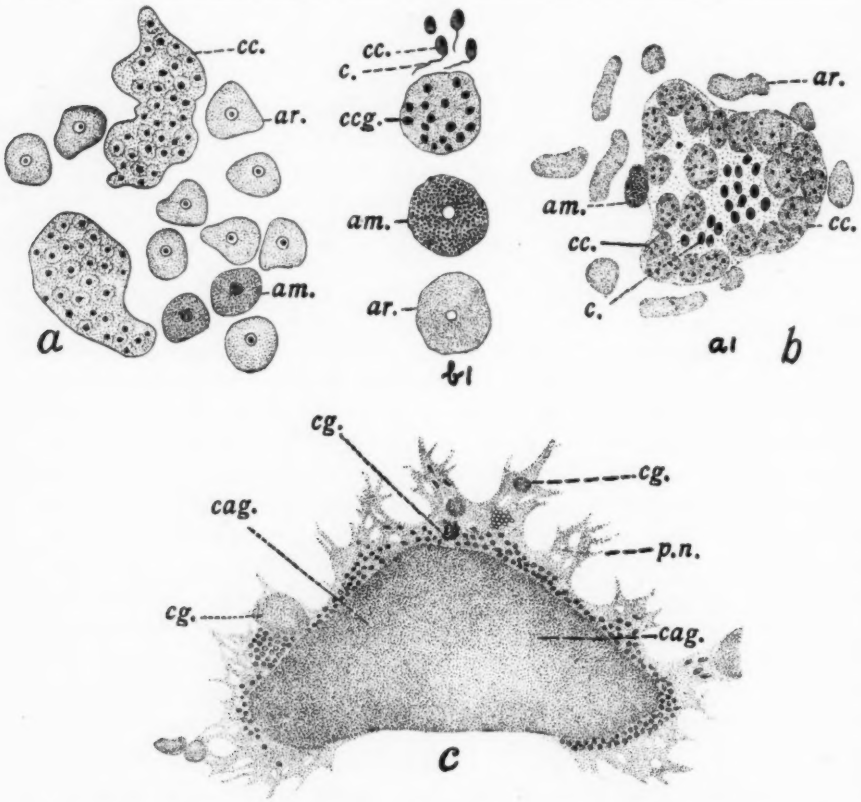
to make a bag containing the pieces of the sponge. This bag was gently squeezed between the arms of a small pair of forceps and the broken down cells were allowed to fall into a finger bowl of clean sea water, so as to form an even layer at its bottom. The water above the layer was decanted, and the remaining fluid with the sponge cells was transferred to petri-dishes, which were then gently lowered to the bottom of an aquarium with suitable arrangements for continual change of water. In a few experiments the dissociated cells were directly squeezed out through the muslin cloth into petri-dishes kept at the bottom of the aquarium tanks. The sponge regenerates thus formed remained as spheroidal restitution bodies, and did not show a tendency to fix themselves or grow to larger sizes. Improved methods of aeration of water and change of different substrata to induce fixation and growth were tried with little success. The restitution bodies retained their bright violet colour throughout the period of thirty days they were reared in the Laboratory. The regenerates were fixed in Bouin's and Zenker's fluids. Sections were stained in Iron Hæmatoxylin counterstained by Orange G. or Feuchsin S., or in Mann's Methyl Blue Eosin. Intra-vitam staining with Methylene Blue, Neutral Red, Tolouidin Blue and Methyl Green was tried with success.

The Composition of the Expressed Material

The pressed out sponge material consists of three types of cells (Fig. 7 *a*). The first type is recognised by the clear cytoplasm and the characteristic vesicular nucleus with a thin nuclear membrane. The nucleolus is prominent with a clear space around it. Owing to their capacity to transform themselves into other cell elements and their power of resistance to adverse conditions, the first type of cells has been termed 'archæocytes'. The second type termed 'amœbocytes' has denser cytoplasm in which lie the nucleus and the nuclear reticulum with the chromatin granules. In a few of the cells of this type one of the chromatin granules appears bigger than the others forming a nucleolus. The third type is the choanocyte (collar cell). Usually the collar cells are found in small groups. When separate and scattered they are easily recognised by their smaller size or the flagellum or both. When they are in groups the flagellæ are completely withdrawn but their small darkly staining nuclei enable them to be traced as distinct entities. In the expressed material the third cell type, *i.e.*, the choanocyte, preponderates. Next comes the archæocyte.

The different kinds of cells mentioned above behave differently with various intra-vitam stains (Fig. 7 *b*). The results obtained are tabulated as follows;—

Stain used	Archæocyte	Amœbocyte	Choanocyte
Methylene blue ..	Takes a uniformly pale green colour leaving a central small space	Stains uniformly pale green with deep blue granules, leaving a central small space	Not stained except for a violet spherule
Neutral red ..	Takes a brick-red colour all over	Uniformly brick-red colour with red granules	Unstained except for a brown inclusion
Tolouidin blue ..	Uniformly greenish blue	Uniformly greenish blue with blue granules	Inclusion alone stained pink
Methyl green ..	Does not take stain	Green granules in an unstained cytoplasm	Does not take stain

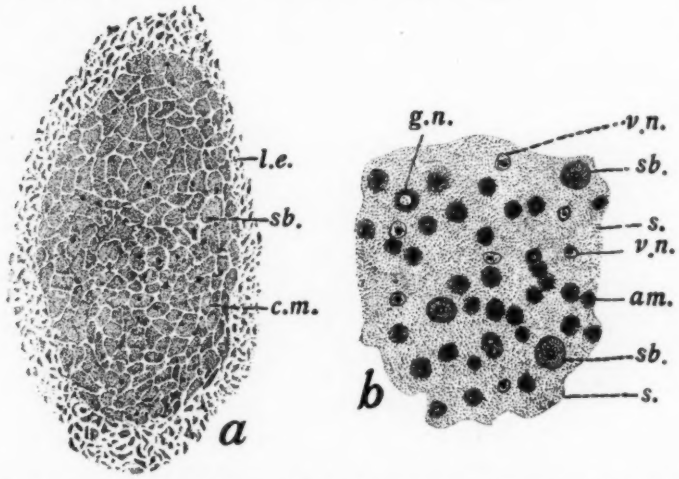


TEXT-FIG. 7. *Callyspongia diffusa.*—(*a*) Cells in the expressed material, 10×90. (*b*) *a* 1 Cells joining together immediately after squeezing out, 10×90; *b* 1. Different cells, 20×90. Both *a* 1 and *b* 1 are intra-vitam preparations with Methylene blue. (*c*) A central large mass drawing smaller masses towards it, 15×8.

am., amœbocyte; *ar.*, archæocyte; *c.*, flagellum; *cc.*, choanocyte; *ccg.*, group of choanocytes; *agc.*, large central aggregate; *cg.*, small cell aggregate; *p.n.*, protoplasmic network.

The Method of Reunion

The cells in the dissociated material are all in a jumble. At first they form an even layer at the bottom of the petri-dish. At this stage the amœboid activity starts, adjacent cells clustering together (Figs. 7 *b*, *a* 1). The collar cells do not move but their pseudopodia when in contact with those of neighbouring cells form cell aggregates, with protoplasmic interconnections. At the end of a few hours, the cell aggregate has the appearance of a reticulum of protoplasmic strands. This reticulum retains the bright violet colour. In about twelve hours' time the amœboid activity of the cells ceases and further growth of the regenerates is achieved by the tugging action of the protoplasmic strands around the cell aggregates (Fig. 7 *c*, Pl. XVII, Fig. 9). At the end of about eighteen hours the reticular strands contract and consequently become short and thick. Portions of this strand get themselves rounded off into spherical, oval or ellipsoidal shape. In twenty-four hours the petri-dish is covered with a large number of such regenerates, 1 to 3 mm. in diameter. Innumerable smaller regenerates about ¼ mm. in diameter are scattered between the larger ones.



Text-Fig. 8. *Callyspongia diffusa*.—(*a*) Sagittal section of regenerate of one days' growth, 7×40; Section of one-day old regenerate in the region of the epithelium, 10×90.

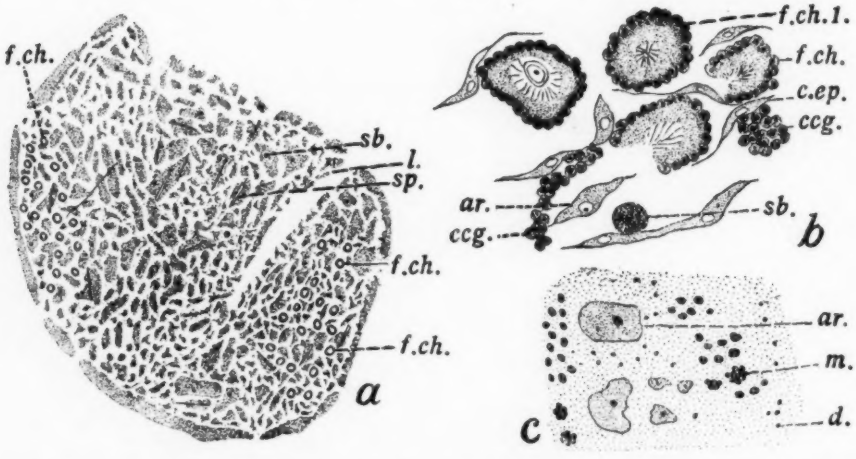
cm., compact interior mass of cells; *g.n.*, granular nucleus; *l.e.*, loose expterior; *s.b.*, spongoblast; *v.n.*, vesicular nucleus; *s.*, incomplete syncytial epithelium formed from archæocytes.

Sagittal section of a regenerate one day old shows the following features: (Fig. 8 *a*). The more abundant cells in the interior which consists of collar cells and amœbocytes are compact while the peripherally lying

archæocytes are less so and have very clear and hyaline cytoplasm. An incomplete external investing layer for the entire regenerate has been formed by the latter. This epithelium has nucleoli in addition to nuclei (Fig. 8 *b*). In the closely packed interior of the regenerate, cell boundaries are very difficult to make out as the cells have formed a syncytium. In their movement from the interior to the exterior a large number of archæocytes assume a fusiform shape.

In sections of regenerates of three days' growth the differentiation of the cells has proceeded still further. The epithelium is incomplete with its cytoplasm appearing more hyaline and the nuclei without any nucleoli. Spaces are developed internally at the points where the epithelium is incomplete. Groups of collar cells with small spaces in between lie apart from the other cells. The amœbocytes take active part in the production of skeletal structures. Amœbocytes having small granules come together to form a beaded string of spongin. Spicules are formed from other amœbocytes, appearing at first as intracellular inclusions.

Sagittal sections of advanced regenerates of seven days' growth show the following features: (Fig. 9 *a*). The epithelium has assumed the definite



TEXT-FIG. 9. *Callyspongia diffusa*.—(*a*) Section of regenerate of seven days' growth, 7×40. (*b*) Part of a section of regenerate of seven days' growth, 20×90; Section of an unhealthy regenerate, 15×90.

ar., archæocyte; *ccg.*, groups of choanocytes; *c.ep.*, canalar epithelium; *d.*, decadent choanocyte nucleus; *f.ch.* 1, flagellated chamber in formation; *f.ch.*, flagellated chamber; *l.*, lacunar spaces; *m.*, dividing archæocyte; *sb.*, spongoblast; *sp.*, spicules.

characters of the adult, the cytoplasm is alveolar and hyaline, and the nucleus had lost its nucleolus. The cell boundaries disappear giving rise to a syncytium. Between the gaps in the epithelium and the syncytium distinct tunnel-like spaces are developed lined by a canalar epithelium built up by the archæocytes. The linings near the chambers differentiate and attain the condition observed in the adult. The small spaces formed earlier inside the collar cell groups are now larger and more distinct, while the choanocytes themselves develop collars and cilia, and the spongoblasts and scleroblasts are actively forming skeletal structures (Fig. 9 *b*). It is noteworthy that the flagellated chambers are developed more in the peripheral regions of the regenerate than in the centre.

Some of the regenerates which showed a tendency to attach themselves to the substratum lost gradually their violet colour and healthy appearance. Sagittal sections of such masses about six days' old exhibited the following characteristics (Fig. 9 *c*). The cytoplasm rarely has any cell boundaries, and the regenerates which take vital stains reveal that the independent cells are of the nature of archæocytes. In the cytoplasm, the numerous, minute, darkly stained granules seem to represent decadent collar cell nuclei. A few of the independent cells are divided into a number of daughter cells, which gradually assume the size of the mother cells. The nuclei in all of them are not vesicular but have instead a rounded darkly staining nucleus of the size of the nucleolus as in the vesicular nucleus, which may be due to the process taking place in the regenerates. The general fate of the daughter cells is, however, one of decay due probably to their being smothered in a slimy secretion.

General

Galtsoff (1925) has fully described the fate of the five kinds of cells involved in the process of regeneration in *Microciona*. According to him the dissociated cells do not become dedifferentiated, but each cell type retains its distinctive characteristics. Wilson and Penny (1930) however recognised only three types of cells in the pressed out material of *Microciona*, namely, the nucleolate mesenchyme cells, gray cells, and collar cells, the functions of which they describe in detail. De Laubenfels (1934) describes in *Iotrochota birotulata* two types of amœbocytes, the fate of which he traced in the formation of new sponges without intermediate dedifferentiation and respecialisation of the dissociated cells. Huxley (1911) found in *Sycon* only collar cells and amœboid cells, of which the former persisted to form the collared epithelium of the regenerate. Muller (1911) and Maas (1907, 1910) found that the collar cells of the parent sponge were absorbed and that new

flagellated chambers in the regenerate were formed from amœbocytes. In *Callyspongia diffusa* the pressed out material consists of archæocytes, amœbocytes, and choanocytes. The archæocytes form the dermal epithelium of the regenerate. The amœbocytes give rise to the scleroblasts and spongoblasts. The collar cells remain in a temporarily dedifferentiated state but resemble to form the flagellated chambers of the adult. The persistence of original cell types applies only to the collar cells. The process by which the dissociated cells come together has been observed differently by various authors. De Laubenfels (1932) described how the dissociated cells were assisted by a hyaline ground mass of slime to approach one another during reunion, in the process of shrinkage. Although amœboid activity of the cells plays some part in the beginning, effective aggregation is achieved by the contraction of the investing slime. Wilson and Penny (1930) stated that reunion was brought about by pseudopodial activity in single cells as well as in small restitution bodies. According to De Laubenfels (1932) amœboid activity alone cannot achieve reunion. In *Callyspongia diffusa* single cells and small cell groups unite by pseudopodial contact. But the protoplasmic network formed around the aggregates brings together smaller aggregates by its activity. In expanding, the network engulfs smaller aggregates but in contracting takes them nearer the central mass.

The tendency of even the healthy regenerates of *Callyspongia diffusa* in the Laboratory aquarium to remain unattached appears to be a common feature of such experiments. Huxley (1911) obtained restitution bodies of *Reneira*, which though alive for a number of days, failed to settle down and grow, while Galtsoff (1933) recorded the tendency of small aggregates to remain unattached. Even in Hydroids which belong to a phylum higher in the scale of evolution, De Morgan and Drew (1914) obtained only restitution bodies from dissociated cells.

Summary

1. The dissociated cells of *Callyspongia diffusa* consist of three types of cells, *viz.*, the archæocytes, the amœbocytes, and the collar cells.

2. The reunion of cells is achieved in the beginning by pseudopodial activity of the cells and later by the formation of a protoplasmic network drawing in adjacent smaller masses by contraction.

3. The archæocytes give rise to the dermal and canalar epithelium.

4. The amœbocytes are concerned in the formation of skeletal structures.

5. The collar cells give rise to the flagellated chambers of the regenerate.

6. The restitution bodies remain alive for about thirty days and do not attach themselves to the bottom.

2. NOTES ON THE EARLY DEVELOPMENT OF *TEDANIA NIGRESCENS* AND *HIRCINIA* SP.

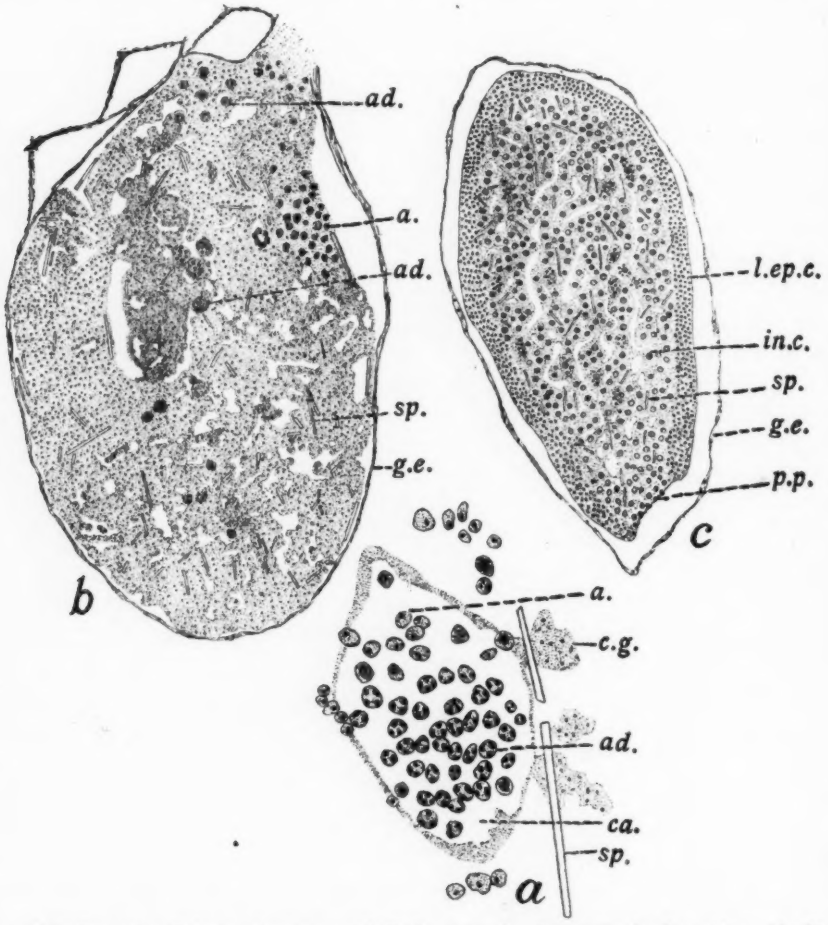
In the following account a short description is given of the early stages in the development of *Tedania nigrescens* and *Hircinia* sp. collected from boulders and rocks underneath the Pamban Railway Bridge, South India.

Early Stages of *Tedania nigrescens*

A study of the early stages was possible from sections of the sponge containing embryos fixed in Zenker's fluid and stained in Iron Hæmatoxylin. Such sections failed to reveal, however, the presence of flagellated chambers in the sponge trabeculæ. The mesenchyme consists of two kinds of cell elements, one bigger in size but fewer in numbers than the other. The larger cell is 6 μ in diameter and has a darkly staining nucleus 2 μ in diameter in the middle of a dense cytoplasm. The other cell type is found in groups of two or more cells with their cytoplasmic boundaries indistinct (Fig. 10 *a*). In certain vacant spaces in the mesenchyme, the larger cells accumulate and begin to multiply in numbers. Their nuclei divide into three or four daughter nuclei amitotically which is followed by cytoplasmic division. This gives rise to a large number of cells. Fig. 10 *b* and Pl. I, Fig. 10 shows an advanced stage in which the multiplication of the cells has gone ahead and the surrounding sponge epithelium is in the process of investing the exterior of the embryo. Though it was difficult to make out cellular details in most of the sections, in favourable preparations such details were made out. The gemmule is composed of a large number of tiny cells in which definite nuclei and cell boundaries can be traced. Wilson (1894) was not able to make out any cellular details owing to the whole mass being darkened by the stain. In the growing gemmule a few of the cells are seen in a stage of amitotic division. Large numbers of parent spicules are also seen enclosed inside the gemmule. Fig. 10 *c* shows a differentiating gemmule lying inside the envelope. Definite cell boundaries are not visible although groups of cells are found here and there. Towards the margin the nuclei are smaller, arranged in four or five tiers and more darkly stained. These nuclei are not seen at one end. It is presumed that they give rise to the larval epithelial cells by organising cytoplasm around them and developing the cilia. The region devoid of them may give rise to the posterior pole of the larva.

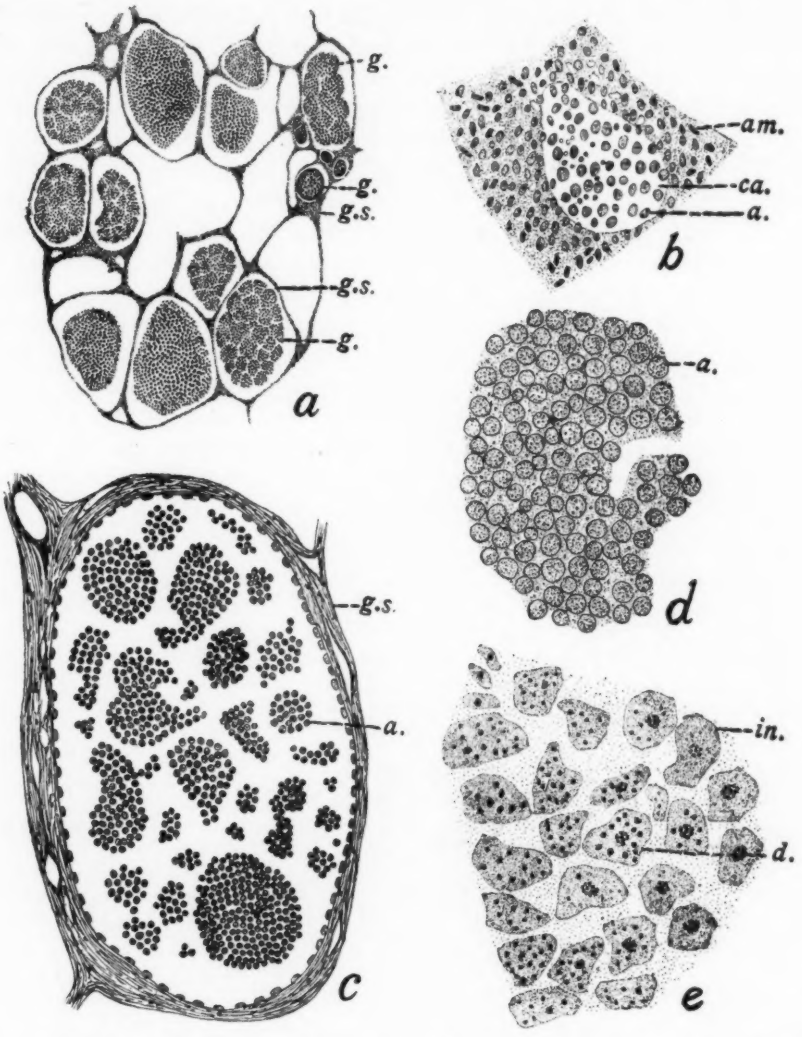
EARLY STAGES IN THE DEVELOPMENT OF *Hircinia* SP.

Pieces of the sponge-containing embryos were fixed in Zenker's fluid and Flemming's fluid. Sections were stained in Iron Hæmatoxylin and Mann's Methyl Blue Eosin. As the embryos were confined to certain parts only of the sponge mesenchyme, the different stages in the development could be studied only in sections passing through that particular region of the sponge (Fig. 11 *a* and Pl. XVII, Fig. 11). The aggregation of numerous



TEXT-FIG. 10. *Tedania nigrescens*.—(*a*) Early stage in the gemmule formation, 10×90. (*b*) Section of developing gemmule, 10×40. (*c*) Section of differentiating gemmule, 7×40.

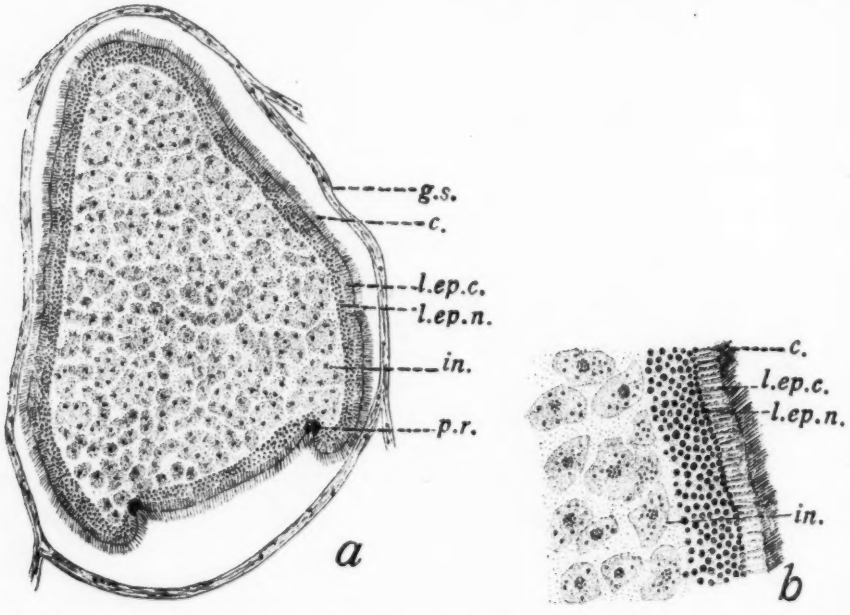
a., Cell of the bigger type concerned in a gemmule formation; *ad.*, amitotically dividing cell of the bigger type; *ca.*, cavity formed in the sponge mesenchyme to lodge the growing gemmule; *cg.*, cell groups of the smaller size; *g.e.*, gemmular envelope; *inc.*, cells of the interior in the differentiating gemmule; *l.ep.c.*, larval epithelial cell; *p.p.*, posterior pole; *sp.*, spicules.



TEXT-FIG. 11. *Hircinia* sp.—Various stages in the gemmule formation: (*a*) Section of the sponge mesenchyme passing through the region of gemmule growth, 10×8. (*b*) early stage in the gemmule formation, 10×40. (*c*) Section of developing gemmule, 10×40. (*d*) Cells inside the gemmule assuming round shape, 10×90. (*e*) Section of gemmule showing differentiation of the internal cells, 10×90.

a., cells inside the chamber formed for the growth of the gemmule; *am.*, amœbocytes moving towards the chamber; *ca.*, chamber; *d.*, cells showing amitotic division; *g.*, gemmule; *g.s.*, gemmular sheath; *in.*, cell of the differentiating gemmule showing division of nucleus into bits.

mesenchyme cells at a common point is the first indication of the formation of gemmule (Fig. 11 *b*). Whether the cavity lodging the developing gemmule is formed in the mesenchyme for the purpose or is a mere space in the sponge mesh work cannot be definitely stated. During their sojourn into the chamber so formed the (mesenchyme ?) cells assume an elongated shape due to compression between cells but soon resume the rounded shape in the cavity (Fig. 11 *c* and *d*). The cells in the cavity increase in number by the migration of similar cells from the surrounding areas. Meanwhile the surrounding tissue shrivels up and gives rise to a multicellular envelope (Pl. XVII, Fig. 12). Fig. 11 *b* shows a small group of round cells which lie in the cavity. When a sufficiently large number of cells has aggregated in the cavity, fusion of adjacent cells commences until a continuous mass is formed. This mass gets resolved into irregular smaller masses each containing more than one nucleus (Fig. 11 *e*). Towards the margin of the differentiating gemmule smaller cells are formed which later transform themselves into the larval



TEXT-FIG. 12. *Hircinia* sp.—(*a*) Longitudinal section of the larva lying inside the multicellular investment, 7×40. (*b*) Section of larva showing part of the inner mass and larval ciliated epithelial cells, 10×90.

c., cilium; *g.s.*, gemmular sheath; *in.*, cells of the inner mass of the larva; *l.ep.c.* larval ciliated epithelial cell; *l.ep.n.*, nucleus of the larval epithelial cell; *p.r.*, pigment ring.

ciliated epithelial cells (Figs. 12 *a* and *b*). The ciliated epithelial cells cover the entire surface of the larva so that a non-ciliated posterior pole is not present. A pigment ring is also developed with normal cilia in this region. The inner mass is composed of a large number of cells with the interspaces probably filled with some fluid. The strong eddies created in the cavity by the ciliary action of the larva presumably breaks open the investing envelope liberating the larva to the outside through the excurrent canal and osculum.

Summary

The origin of a ciliated larva from the gemmule of a horny sponge (*Hircinia* sp.) is recorded here for the first time. The larval development resembles that of *Esperella fibrexilis* (Wilson, 1894) and of the Hexactinellids *Ventrollula fertili* and *Leucopsacus orthodocus* (Ijima, 1901).

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Explanation of Plate XVII

(Figs. 1 to 9 pertain to *Callyspongia diffusa*)

Fig. 1. Photograph of the sponge.

Fig. 2. Photograph of sponge pieces filled with sexual reproductive bodies in various stages of growth.

Fig. 3. Section of sponge showing formation of multinucleate masses in the sponge mesenchyme (10×40).

Fig. 4. Section of fully formed gemmule in the sponge body (7×40).

Fig. 5. Section of differentiating gemmule where the collar cells have begun migration to the periphery (10×40).

Fig. 6. Section of sponge showing fully formed larva inside gemmular envelope (10×8).

Fig. 7. Photograph of the swimming larva (7×40).

Fig. 8. Section of pupa just attached (10×40).

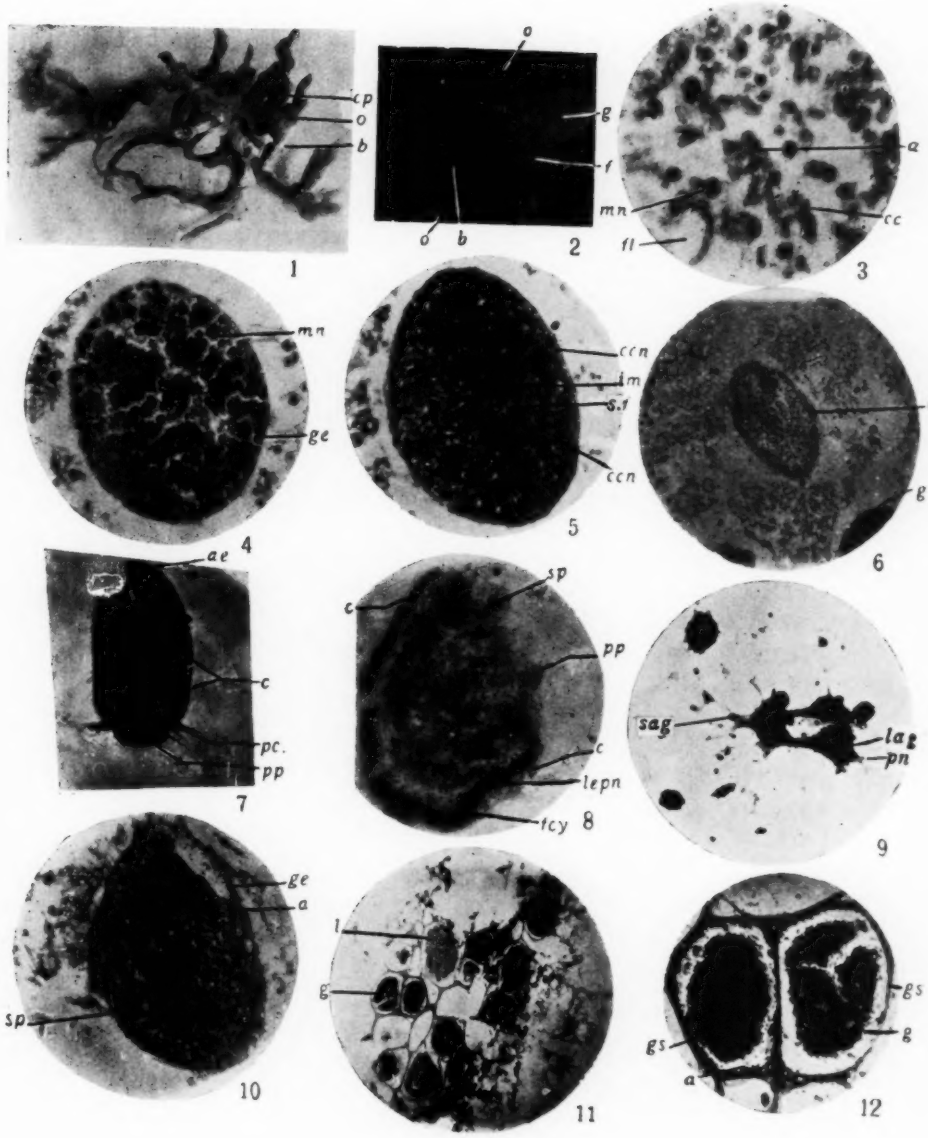
Fig. 9. Photograph showing formation of regenerates after dissociation of cells (5×40).

Fig. 10. Section of developing gemmule of *Tedania nigrescens* (10×40).

Fig. 11. Section of *Hircinia* sp., showing various stages in the development of gemmules in the sponge body (5×40).

Fig. 12. Section of *Hircinia* sp., showing the development of two gemmules in the chambers formed in the mesenchyme (10×40).

a., amœbocyte; *b.*, encrusting base; *c.*, cilium; *cc.*, choanocyte; *ccn.*, choanocyte nucleus; *cp.*, conical process; *fl.*, flagellated chamber; *fcy.*, fyused cytoplasm of the larval epithelial cells; *f.*, gemmule forming plasmodium; *g.*, gemmule; *ge.*, gemmular envelope; *in.*, cells of the inner mass; *l.*, larva; *mn.*, multinucleate mass; *o.*, osculum; *pc.*, cilia of the pigment ring; *pp.*, posterior pole; *pn.*, protoplasmic network; *sag.*, small aggregate; *sf.*, spongin fibre; *sp.* spicule; *ae.*, anterior end; *lag.*, large aggregate; *lepn.*, nucleus of the larval epithelial cells.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SOME BURROWING FISHES FROM TRAVANCORE*

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CONTENTS

		Page
1.	Introduction	311
2.	Description of Species and Bionomics	311
3.	General Considerations..	320
4.	Structure of the Eyes	321
5.	Cutaneous Organs	323
6.	Summary	327
7.	Acknowledgements	327

Introduction

Fishes with a burrowing mode of life have been objects of interest as is evident from the writings of Norman (1931), Hora (1935 and 1936), Rao (1939) and Das (1946). In these fishes the shape of the body, the structure and function of the eyes are seen to have been influenced by their peculiar habit. The present paper deals with four species of fishes from Travancore which show a burrowing habit. The species dealt with are *Amphipnous fossorius* sp. nov., *Symbranchus bengalensis* (McClelland), *Tænioides cirratus* (Blyth) and *Trypauchen vagina* (Bloch and Schneider), the first two belonging to the family Symbranchidæ and the other two to the family Gobioidæ. Of these the first one appears to be new to science. In all cases I have attempted to give the detailed bionomics of the forms concerned and in the case of the first which appears to be new the specific characters also. Though the families are far apart systematically, still their adaptations to a burrowing mode of life have been on similar lines.

Description of Species and Bionomics

Genus *Amphipnous* Müller

Amphipnous fossorius sp. nov. (Fig. 1)

Body eel-like, length of head being contained 9–10 times in the distance between the head and the anus; length of tail 4–4½ times in total length.

* Part of Thesis for which the Degree of Master of Science was awarded by the University of Travancore in 1947.

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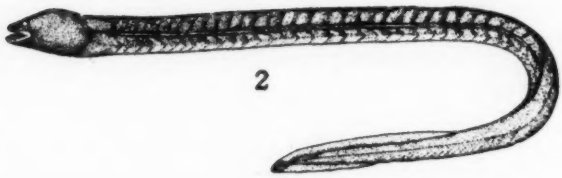

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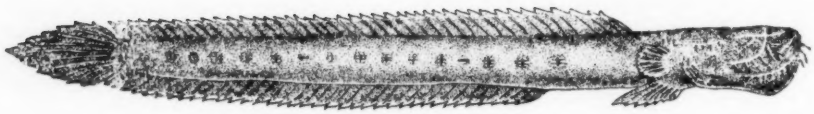

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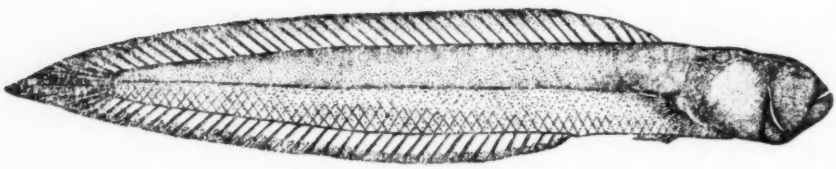

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FIG. 1. *Amphipnous fossorius* sp. nov.
FIG. 2. *Symbranchus bengalensis* (McClelland).
FIG. 3. *Tænioides cirratus* (Blyth).
FIG. 4. *Trypauchen vagina* (Bloch and Schneider).

Paired fins entirely wanting; eyes very small, three to four diameters from end of snout, situated in the anterior one-fifth of the head midway betwen the end of the snout and posterior extremity of the jaws; and covered over by a thick semi-transparent skin. There is a nostril above each orbit slightly above the anterior border. Upper jaw is somewhat longer than the lower and the lips are fleshy. Teeth are present on the premaxillæ, maxillæ and palatines. Maxillary teeth are sharp, pointed and recurved, arranged in two or three rows at the anterior end, but posteriorly the rows converge into a single row. Near the symphysis of the upper jaw some of the premaxillary teeth are disposed in the form of two ridges one on each side having an antero-posterior direction. The palatine teeth are comparatively thicker than the maxillary teeth and possess bluntly pointed tips. They are arranged in a single row posteriorly, but spread out anteriorly in a double row. On

each ramus of the mandible there are three to four rows of pointed recurved teeth similar to the maxillary teeth in size and shape.

There are six branchiostegals and three gill slits of which the anterior two are very distinct. None of the branchial arches carry well-developed gill filaments. Gill membranes almost grown over the isthmus, presenting a single crescent-like transverse opening on the ventral side. Opening into the branchial chamber is a flask-shaped respiratory air-sac on each side and these extend backwards. The narrow neck-like part of the sac is directed forwards and each sac lies over the corresponding row of gill slits, occupying nearly the whole of the branchial region. The sacs which are highly vascular are visible when the skin in the neck region is removed.

The dorsal fin commences from a point midway between the anus and posterior tip of the body and posteriorly it is confluent with the anal fin. The anal fin reaches forward only half the length of the dorsal. In other words the dorsal and anal fins are continuous and there is no separate caudal fin. The fins are very narrow and are devoid of fin rays.

The body is covered over by minute cycloid scales which are arranged in distinct longitudinal rows.

There are 126–129 vertebræ of which 73 are precaudal and 53–56 caudal. Thus it will be seen that while the precaudal vertebræ are constant, the number of caudal vertebræ is variable and they are also much flattened. A distinct lateral line extends from the head to the posterior tip of the body.

The maximum length of the specimens so far collected by me is 230 mm. and one of them which was 195 mm. was found to be sexually mature.

Measurements of specimens in mm.

Total length ..	229·6	226·0	198·4	192·0	189·5
Length from anus to posterior end of the body	49·9	52·9	49·9	45·7	41·9
Length of head ..	17·7	17·0	17·5	14·7	15·2
Diameter of the eye ..	1·0	1·0	0·9	0·9	0·9

The possession of six branchiostegals, nature of the gill membrane, the gill arches with rudimentary gill filaments, the presence of respiratory air-sacs in the branchial region and the longitudinally arranged scales on the body go to show that this fish belongs to the genus *Amphipnous* Müller.

The only species of this genus so far recorded from India is *Amphipnous cuchia* (Ham. Buch.).

The number of vertebræ is 73|53–56 and the dorsal fin-fold commences midway between the level of the anus and the posterior end of the body. Palatine teeth are in a double row anteriorly and mandibular teeth are in two or three rows. The colour is reddish brown dorsally and deep red towards the ventral aspect, without any colour markings.

The above characters indicate many important points of difference of the present species from *Amphipnous cuchia* (Ham. Buch.), as described by Day (1876). The latter is reported to reach a length of two feet, with 106|65 vertebræ, the dorsal fin commences from a point slightly anterior to a vertical line drawn through the anus, the palatine teeth and mandibular teeth are only in single rows and the colour is greenish or chestnut brown becoming lighter towards the abdomen and there are numerous colour markings on the body.

The above differences have been confirmed by me by a comparison of the present species with specimens of *Amphipnous cuchia* (Ham. Buch.) in the collection of the Zoological Survey of India.* I consider there is sufficient justification for describing this as a new species. Because of the burrowing habit the specific name *Amphipnous fossorius* is proposed for the present form.

Type specimens of *Amphipnous fossorius* sp. nov. are deposited in the Marine Biological Laboratory, Trivandrum, Travancore.

Amphipnous fossorius sp. nov. was collected in 1942 in Travancore by Dr. C. C. John, formerly Professor of Marine Biology and Fisheries in the Travancore University. He obtained it from the marshy areas in the paddy fields and banana plantations adjoining the Karamanai river as it passes down Karamanai, a village near the city of Trivandrum. The fish is found in the slushy mud in between the plantations at about a depth of two to three feet. The fish has almost abandoned its aquatic habitat and taken to a burrowing mode of life as will be seen by its structural adaptations.

Examination of the stomach contents showed that its food consisted of small worms found in the soil. Mature specimens were found during the rainy season and this probably gives some clue to their breeding season.

At this stage it may be worthwhile to compare the habits of this fish with that of *Amphipnous cuchia* (Ham. Buch.). It is described that like other fishes *A. cuchia* is an inhabitant of fresh and brackish waters of India and

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* I take this opportunity to thank Dr. B. N. Chopra, formerly Officiating Director, Zoological Survey of India, for kindly permitting me to examine the collections in the Zoological Survey, when I visited that in 1945.

Burma, spending much of its time in the grass on the banks of ponds like a snake. Das (1927) who studied the bionomics of this fish in great detail observes that it spends a greater part of its life outside water wriggling along the muddy banks of rivers and ponds, in grassy meadows and muddy places among the roots of bushes. According to him when the dry season approaches it burrows down in the banks of streams and here in the same burrow the male and female pass the summer together. Hora (1935) states that it is usually found lurking in holes and crevices in the muddy banks of marshes and slow running rivers and that it is particularly abundant in the *bhils* of the deltaic districts of Lower Bengal. He agrees with Das (*op. cit.*) that it is often found wriggling about in wet grass several yards away from water. Das (1946), who made a further study of the ecology, bionomics and life-history of *A. cuchia*, says that it is a semi-terrestrial fish which habitually spends its time in wet mud and often makes 'U'-shaped or zig-zag burrows in the soft mud in which it lives. These burrows are provided with entrance and exit holes which are often mistaken for and compared with those of fresh-water crabs. He has further shown that eggs are laid in the burrows and that young ones that hatch out spend their early part of life in the parental burrows.

Other observations.—Amphipnous fossorius sp. nov. occurs in regions far beyond the limits of tidal influence in the neighbourhood of small streams where the soil is soft and marshy. The only source of oxygen therefore for this fish is either atmospheric air or air present in the moist mud surrounding it. This may probably explain the presence of well-developed air-breathing organs and the reduction of gills in this fish. The air-breathing organs consist of a pair of air-sacs situated one on either side in the neck region and these function almost like the lungs of higher vertebrates though there is no homology between the two. Air is taken in through the mouth into the air-sacs which as a result become conspicuously inflated. The air-sacs which are highly vascular perform the gaseous exchange and the used up air is expelled through the midventral gill opening exactly as in *Amphipnous cuchia* [Das (1927), pp. 197–99]. From a careful study of the habit of this fish it is seen that this mode of respiration is possible only when the fish is nearer to the surface where there is possibility of direct access to air. But when the fish burrows deeper its respiratory difficulties will increase. Under such conditions gill respiration and aerial respiration through the air-sacs are difficult. Possibly the skin acts as an accessory organ of respiration.

Cutaneous respiration is considered a normal process in many forms of vertebrates both terrestrial and aquatic. Spallanzani (1803) first demon-

strated that the frog by means of respiration through the skin continued to live for a long time in air after the extirpation of the lungs. Berg (1886) confirmed this. The existence of cutaneous respiration in fishes also has been suggested by Provencal and Humboldt (1811). Quincaud (1873) found that an eel of 530 gm. weight absorbs 0·58 c.c. of oxygen in an hour through the skin. This according to Norman (1931) is "not improbable as it sounds. Since respiration is nothing more than an exchange of gases by the blood, there is no reason why these gases should not penetrate through the thin membrane-like the skin and reach the blood contained in the fine vessels situated therein."

The possibility of cutaneous respiration in *Amphipnous fossorius* is suggested not only by its particular habit of dwelling underground but also by a study of the structure of its skin, which, especially on the ventral side, is pink and is devoid of pigments. The epidermis is comparatively thin. Beneath this there is a sub-epidermal layer of spongy tissue followed by a thin layer of connective tissue. In the sub-epidermal layer there is a network of blood vessels and capillaries lying immediately below the epidermis. These blood-vessels are separated from the surrounding medium only by a thin epidermis and their close proximity to the epidermis indicates that these vessels are probably respiratory in function. The deepening of the colour of the skin under certain conditions probably occurs when more blood rushes into these superficial vessels for absorption of oxygen.

We find in this form as in *Amphipnous cuchia* (Ham. Buch.) a very marked degeneration in the structure of the gills. As in the latter, there are only three branchial arches of which the first one is devoid of gill filaments. The second arch carries filaments which are very much reduced in size and number, while the third arch carries only a degenerated vascular membrane. If the animal is kept submerged in water without access to atmospheric air it struggles to breathe with the help of the gills by violently gulping water through the mouth. The colour of the skin deepens showing thereby increased circulation of blood through the skin (probably attempting cutaneous respiration). But both these are of no avail and the animal collapses in about 3 hours, clearly indicating the inadequacy of both cutaneous and gill respiration alone in the absence of direct access to atmospheric air.

In *Amphipnous fossorius* sp. nov. the body is fusiform, cylindrical and snake-like and devoid of appendages with pointed snout and tail. Even the median fins which are devoid of fin rays are represented only by a narrow fold of skin. This fish is definitely adapted to a burrowing mode of life.

Genus *Symbranchus* Bloch.

Symbranchus bengalensis (McClleland).

Symbranchus bengalensis Day, 1876.

Body eel-like, length of head 7–8 times in the distance between end of snout and anus; diameter of eyes 20–25 times in the length of head. Eyes minute placed about midway between end of snout and posterior extremity of the jaws, and completely covered over by a semi-transparent adipose skin. Snout anteriorly rounded, lips fleshy, the upper jaw rather longer; cleft of mouth extending to some distance behind the orbits. Teeth in upper jaw small and pointed, not continuous at symphysis; those in the lower jaw rather larger with a narrow edentulous space between the rows on each side, laterally they are in a single row; palatine teeth in a band.

The anal fin is situated in the last fourth or fifth of the total length and the dorsal fin commences in front of it. Caudal fin hardly conspicuous. A prominent lateral line extends from behind the head to the posterior extremity of the body.

The colour is light brown becoming pale towards the ventral side. Specimens preserved in spirit are light grey in colour.

Symbranchus bengalensis is found in the Kumaragom region of the Vembanad backwaters, in the estuarine region of the Periyar river and in Kuttanad. The bed of the lake is formed of loose clay mixed with humus and organic debris. The fish lives buried in the sub-soil clay at a depth of about one to one and a half feet. If the clay after being dug out is carefully searched it is always possible to collect this fish. In Parur, North Travancore, in the estuarine regions of the Periyar river the fish is found in submerged paddy fields adjoining backwaters. In all localities, referred to above, the water is brackish in summer and fresh in the rainy season.

From an examination of the stomach contents it is evident that the fish is carnivorous, feeding on small prawns, and young fishes.

Unlike *Amphipnous fossorius* sp. nov., the gill arches bear well-developed gill filaments thereby showing that it is capable of aquatic respiration. The fish can however use atmospheric air directly when needed through the vascular epithelium which lines the branchial chamber. The skin is deeply pigmented and blood vessels are not richly developed in it. Here the possibility of cutaneous respiration is very much less.

The fish is an efficient burrower as suggested by the pointed snout and cylindrical snake-like body. Lateral appendages are entirely wanting and

the median fins are narrow and reduced. The fish usually burrows in mud under water. In the inter-tidal regions it probably burrows when the tide recedes and the bed is exposed. The fish can be seen swimming close to the bottom and whenever disturbed it burrows into the soil.

Genus *Tænioides* Lacépède

Tænioides cirratus (Blyth).

Amblyopus cirratus Blyth, 1860.

Amblyopus branchygaster Günther, 1861.

Gobioides cirratus Day, 1876.

Tænioides cirratus Koumans, 1941.

D. VI, 43–47; A. I/42–45; P. 13.

Body cylindrical anteriorly and compressed posteriorly. The head is contained 6–7 times and the caudal fin 10–12 times in the total length. Eyes situated dorsally on the head and are completely covered over by skin. They are very minute and inconspicuous and are visible only if the overlying skin is removed. The head is blunt and possesses prominent ridges. Strong caninoid teeth are present on both jaws. In the upper jaw there is an outer row of four teeth on each side and behind it there is a band of small pointed teeth. A row of three barbels are also found on each side of the chin.

The dorsal and anal fins are separated from the caudal by a deep notch. Caudal fin usually rhomboid. Pectoral small and about three times in the head. A characteristic feature of the fish is the possession of a row of 16–17 peculiar organs on each side along the lateral line region. Similar structures are also present on the ridges of the head and chin region. The fish is deep pink in colour due to a rich ramifying system of blood vessels in the skin.

Tænioides cirratus occurs near the bar mouth of the Vembanad back-waters and in the estuarine regions of the Periyar river. It makes small burrows in the shallow regions within tide limits and occasionally in deep water areas also. Each burrow is a distinct tube with a definite opening on the surface through which the fish can come out and go in whenever necessary. These burrows are very similar to those described by Rao (1939) and are made by biting away mud with the strong caninoid teeth or by shovelling it up with the head. During the former process the mud and water taken into the mouth are expelled through the opercular openings. In these burrows a free circulation of water is maintained and this current helps respiration and also feeding to some extent.

Laboratory observations show that as soon as a fish is introduced into an aquarium tank containing a thick layer of mud, it goes down to the bottom and after swimming about for a few minutes, selects a suitable spot and quickly burrows down into the mud with its head.

The burrows are found in shallow water within tidal influence. The fish appears to come out of its burrow only at night when it swims about freely in search of prey. They are often dislodged from their burrows and are carried out by the receding tide and then are caught in large numbers in stake nets.

The natural food of the animal consists of small prawns, copepods and small fishes. The fish is not much esteemed as food.

The possession of well-developed gills suggests that the fish normally performs gill respiration. But the fish is capable of living out of water for a considerable time by taking air into the branchial chambers. This air-breathing habit is particularly helpful for a fish of this kind as it is liable to get exposed and stranded during receding tides.

The skin of the fish is soft, unpigmented laterally and ventrally. It covers the body like a loose jacket as in the Amphibians. It is pink in colour which intensifies when disturbed or when the animal is taken out of water. The epidermis consists of three or four layers of flattened cells of uniform size and in the sub-epidermal layer there is a rich ramifying system of blood vessels. In live specimens this ramifying system of blood vessels is visible through the thin and semi-transparent skin. Describing a similar burrowing fish *Typhlogobius californiensis*, Ritter (1893) suggests that the absence of pigment on the ventral surface and the existence of an intricate system of blood vessels in the sub-epidermal layer may indicate the possibility of cutaneous respiration. According to him though the most serious objection to the supposed respiratory function of the skin lies in the thickness and density of the epidermis and the fact that the entire surface is thickly beset with slime secreting cells, it is not likely that the epidermis in this case would offer greater resistance to the interchange of gases than that of the frog.

Genus *Trypauchen* Cuv. Val.

Trypauchen vagina (Bloch and Schneider).

Gobioides vagina Bloch and Schneider, 1801.

Trypauchen vagina Day, 1876.

Trypauchen vagina Koumans, 1941.

D. VI, 41–49; A. I|39–46; P. 15–18; L. 1. 80–115; L. tr. 21.

Body compressed, height being contained 9–10 times in length and 11½ in total length. Head is contained 6–6½ in length and 7–7½ in total length. Eyes very small, invisible, sunk much below the surface on the dorsal side of the head and are covered over by thick adipose skin. The occipital crest is slightly elevated. Mouth oblique, lower jaw prominent. Teeth in many rows on both jaws, outer row enlarged and caninoid. The head, nape and breast are naked. Scales on the body cycloid. Dorsal fin continuous, rays of first dorsal feeble, ⅓ depth of body, 6th ray being far removed from the 5th. Length of pectoral ½ of head, lower five rays short and branched. Ventral 2½–3 in head, the disc not emarginate posteriorly. Caudal slightly pointed. The colour of the fish in life is deep pink.

This is also a blind fish with more or less the same habits and habitat of those of *Tænioides cirratus* (Blyth). Specimens were collected from the bar mouth of the Vembanad backwaters, estuarine regions of Periyar river, Kayamkulam backwaters and also from the mud banks off the Alleppey coast. It only buries itself in the bottom mud.

In *Trypauchen vagina* gills are normally developed and aquatic respiration seems to be normal process. As it is likely to be exposed to atmospheric air when the tide recedes in the estuarine areas, the capacity for aerial respiration is also developed. When the fish is taken out of water it is seen to take air into the highly vascular branchial chamber through the mouth. The structure of the skin suggests the possibility of cutaneous respiration. The skin shows complete absence of pigment and the presence of a well-developed system of blood vessels in the sub-epidermal layer. The epidermis is thin. Integumentary respiration apparently supplements gill respiration to some extent.

General Considerations

From a comparative study of the burrowing habits of the fishes described above it will be seen that majority of them burrow into the soil only for protection and safety during unfavourable times. *Symbranchus bengalensis* and *Tænioides cirratus* probably resort to burrowing only when the tide recedes or when otherwise disturbed. *Trypauchen vagina* does not appear to be a burrower but only buries itself in mud. Also in *Tænioides cirratus* and *Trypauchen vagina* the shape and other external features of the body are less snake-like. Such fishes cannot therefore be regarded as true burrowers, because they do not possess all the characteristics of a true burrower. A true burrower is one which may be described as having permanently adapted itself to a sub-terranean mode of life even to the extent of

seeking its food in the soil itself and also laying its eggs in the same environment. When this fact is taken into consideration, among Indian fishes, the genus *Amphipnous* is probably the only instance where burrowing adaptations have developed to the maximum extent. *Amphipnous cuchia* (Ham. Buch.) has been known to lay eggs in burrows (Das, 1946) and though the breeding habits of the new species have not been studied so far its food which consists mostly of small worms in the soil indicates the well-developed burrowing habit of the fish. In this connection I wish to point out that I have taken into consideration only those characters as have to do with a burrowing mode of life. However, all the fishes described in this paper seem to have developed certain common characteristics, the most important of which are accessory respiration as described above and a varying degree of eye degeneration.

Structure of the Eyes

The eye structure in all the forms studied here shows some deviation or other from the normal.

Amphipnous fossorius.—The eye is very small being 200–235 times in the total length, and is covered over by a thick semi-transparent adipose skin. The eye ball is very much sunk inwards and so the outer adipose skin is almost flush with the surrounding skin. Sections of the eye show that in the region where normally the rods and cones occur there are certain scattered structures resembling degenerated cones, between which there is a loose mass of tissue. It is also seen that the pigment layer is a uniform dark brown mass without cell limits. The lens is spherical. Neither a falciform process nor a hyaloid vessel is present and the optic nerve is much reduced.

Symbranchus bengalensis.—The eye though comparatively small and covered over by semi-transparent adipose skin, shows all the different parts of a typical piscine eye, the only difference being the absence of the falciform process or hyaloid vessel.

Tænioides cirratus.—Detailed histology of the eye has already been worked out by Eggert (1931).* In this fish the eye is very minute and is covered over by an adipose skin formed of several layers of epidermal cells followed by a thick layer of connective tissue. The different parts of the eye are much reduced. The sclerotic coat is not quite continuous. Choroid has blood corpuscles in it and thus forms a chorio-capillaris. The retinal

* Eggert described the eye structure of *Amblyopus brachygaster* Günther which according to Koumans (1941) is a synonym of *Tænioides cirratus* (Blyth).

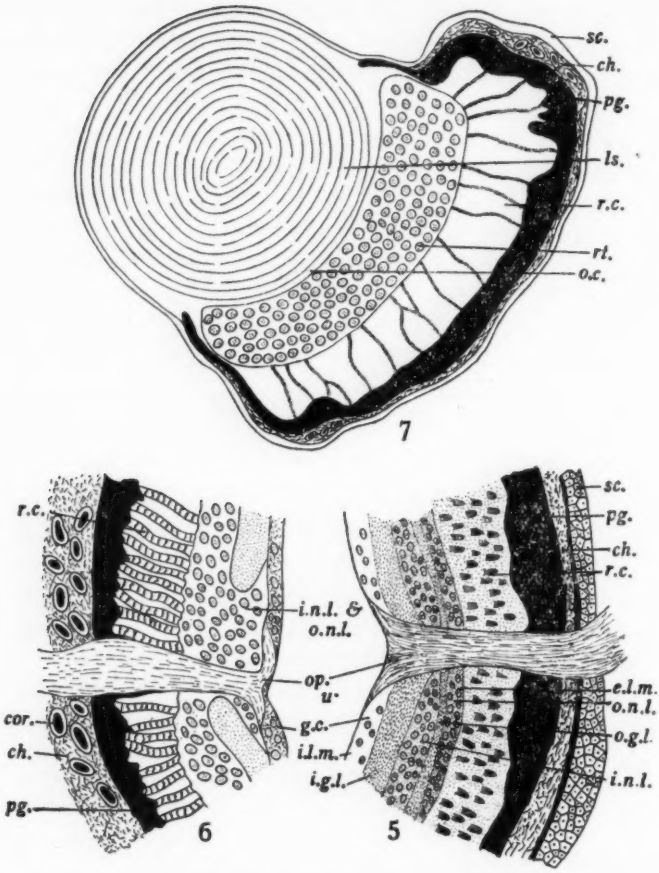

Fig. 5. Part of the retina of *Amphipnous fossorius* sp. nov. : Semi-diagrammatic.
Fig. 6. Part of the retina of *Tænioides cirratus* (Blyth): Semi-diagrammatic.
Fig. 7. Section of the eye of *Trypauchen vagina* (Bl. and Schn.): Semi-diagrammatic. The adipose skin covering the eye is not shown.

pigment is a uniform dark brown mass. The inner and outer nuclear layer of the retina cannot be clearly differentiated and both these layers show only scattered nuclei. Cones could not be made out and rods, much degenerated, are situated wide apart. The optic nerve is very much reduced and ensheathed by pigment to some extent during its passage through the retina. Neither a falciform process nor hyaloid vessel is present.

Trypauchen vagina.—The eye is situated dorsally on the head in a deep pit, covered over by a very thick adipose substance. It is very minute and

shows maximum degeneration. The optic cup is rather shallow. The sclerotic and the chorio-capillaris are very much reduced. The pigment is a uniform dark brown mass and the space between it and external limiting membrane of the retina, *viz.*, the layer of rods and cones does not show any specialised structure. The different sensitive layers of the retina are represented by a mass of degenerated nuclei loosely arranged. Optic nerve is a mere vestige and cannot be easily distinguished. A hyaloid vessel or falciform process is absent.

From what has been described above it will be seen that a burrowing mode of life has affected in one way or other the structure of the eye. The falciform process is absent. There has also been a considerable reduction in the size of the eye which in all cases is covered over by adipose skin. The lens which in normal cases is flush with the level of the head shows signs of being sunk inside the optic cup.

Cutaneous Organs

Mention may be made in this connection of certain specialised organs found on the body surface in *Tænioides cirratus* and *Trypauchen vagina*. In the former they are present on the head and the sides of the body, while in the latter they are confined to the head.

In *Tænioides cirratus* the organs on the head are situated on ridges present on the dorsal and lateral sides and these ridges essentially follow the lateral line system. Those on the sides are distributed in groups in the lateral line region. Each group on the side of the body is surrounded by an elliptical area and to the skin over each elliptical area are attached three powerful muscle bands. The position and arrangement of these organs are well described and figured by Aiyar and Nair (1944) from specimens obtained by them from the Madras Coast immediately after heavy rains.

As regards the structure of these organs it is to be mentioned that those on the head and on the sides of the body are of the same structure, the only point of difference being the absence of muscle bands in the former. Though Eggert (1931) described the organs on the head, he did not mention anything about those on the sides. Each organ consists of a central mass of columnar cells with basal nuclei and granular cell contents, recalling the structure of a typical glandular cell. Surrounding these glandular cells is a group of elongated mantle cells. The organ is supplied by a prominent blood vessel and there are also nerve fibres to enervate it. Sections of these organ on the sides of the fish seem to show an external opening guarded by an ectodermal fold but this point requires further elucidation. The diagram given

by Eggert (*op. cit.*) as well as the photomicrographs given by Aiyar and Nair (1944) illustrate clearly the structure. An external opening for the organ is not mentioned either by Eggert or by Aiyar and Nair. Ritter (1893), while describing similar organs on the head of *Typhlogobius californiensis* got sections of the organ both with and without external opening and in those with an overlapping ectodermal fold there was an excavation on the summit. On these facts he concludes that the organ actually opens to the outside and in those where there is an over-lapping ectodermal fold it may be that the organ is withdrawn into the skin. Ritter does not establish this view definitely and is himself sceptical about it, because the organs he described were devoid of muscles and also in organs which showed an external opening there was the excavation at the summit. In this connection it may be mentioned that the organs on the head of *Tænioides cirratus* are devoid of muscles and they do not show an external opening even though the summit of the organ projects a bit above the body surface. From this one may conclude that the organs on the head being enveloped by the epidermal fold have lost the muscles, while those on the sides are capable of being opened or closed at will by muscle action. In other words the organs on the head are not under the control of the animal while those on the sides are under control as suggested by Aiyar and Nair (*op. cit.*).

Though Eggert (*op. cit.*) described in detail the organs on the head as sense knobs (*sinnes knospen*) he did not attach any special function to them. He did not also mention anything about those on the sides. Aiyar and Nair (*op. cit.*) who were the first to ascribe a definite function to these organs considered them as luminous. They have made it explicit that their conclusion is based solely on the structure and not on the behaviour of these organs. In structure these seemed to conform closely with the luminous organs of other known luminous fishes. On this basis they formed their conclusion and it has to be mentioned that such was the case in most known luminous fishes.

During the course of the present investigation I kept live specimens of *Tænioides cirratus* under observation both during day and night. But on no occasion did I succeed in noticing any production of light. As Aiyar and Nair (*op. cit.*) suggested that luminosity may be a means of luring prey attempts were made to stimulate the fish by dangling small fish which constitute their food, in front of their mouth. In this connection it may be stated that all these experiments were tried at night and in complete darkness. But none of the above attempts succeeded in producing luminosity though all the specimens remained healthy and active throughout the experiment.

Eggert (*op. cit.*) as already stated described these organs as sense knobs without mentioning any particular sensory function to these. Aiyar and Nair (*op. cit.*) described these as luminous organs on the strength of its similarity in structure with the luminous organs of other known luminous fishes. But Ritter (*op. cit.*) in describing similar organs for the fish *Typhlogobius californiensis* considers them as tactile sense organs, though he doubts the efficiency of these in compensating the loss of eye sight in the fish. He also says that similar organs are present in other genera like *Gobius*, *Gobiodon*, *Lepidogobius* and *Clevelandia*. Thus we get a number of fishes with organs of a similar structure and probably similar function.

It has already been stated that in *Trypauchen vagina* also certain specialised organs are present but here they are restricted to the head region. In structure they differ very much from those in *Tænioides cirratus* or *Typhlogobius californiensis*. In *Trypauchen vagina* these organs are described in detail and figured by Eggert (*op. cit.*). Each organ is sunk in a pit which communicates with the outside by a distinct pore and consists of a distinct nerve plate enervated by a well-developed nerve. So here it is almost definite that it is a sense organ. As in *Tænioides cirratus* Eggert (*op. cit.*) calls these also sense knobs but does not mention any sensory function for them.

The association of a prominent blood vessel with each organ and its more superficial position in *Tænioides cirratus* are points which differentiate it from the sense organ in *Trypauchen vagina*. The glandular structure of the organ may lead one to ascribe a secretory function. French chalk was smeared on the body of living specimens. After sometime these organs swelled up and the french chalk over each came off as a single plate. This might suggest a secretory function. But the body of the animal is always slimy due to the presence of a rich supply of cutaneous mucous glands. It is therefore difficult to understand the need for any special mucous glands. Eggert (*op. cit.*) also doubts its secretory activity and says that it is not a mucous secreting gland.

Three functions have thus been suggested for these organs, *viz.*, sensory, secretory and luminous (Ritter, 1893; Eggert, 1931; Aiyar and Nair, 1944), and all these mainly on the morphological structure.

In their characteristic arrangement, one is reminded of the lateral line organs in fishes. Further a lateral line is absent in the fish. They may probably be some modification of the lateral line system. However, further experimental evidence is necessary to elucidate these points.

Statement for Adaptations for a Burrowing Habit

Name of Species	Shape of body	Snout and Tail	Fins	Gills	Air-Sacs	Nature of skin	Eyes	Cutaneous organs
Amphipnous fossorius	Long, cylindrical and snake-like	Both pointed	No paired fins Median fins as narrow folds without fin rays	Very much reduced	Well developed, one on each side in the neck region	Sub-epidermal layer shows a vascular network	Reduced in size. Also retina has a loose mass of tissue with no definite rods and cones	Absent
Symbanchus bengalensis	do	do	do	All gill arches have well-developed gill filaments	Absent	Skin pigmented without a sub-epidermal vascular network	Reduced in size. Almost normal piscine structure	do
Tænioide cirratus	Less snake-like	Snout blunt, tall rhomboidal and pointed	Paired and median fins well developed	do	do	Skin without pigment and has a rich vascular network in the sub-epidermis	Very much reduced with highly degenerated retina and optic nerve	Present on head and sides of body
Trypauchen vagina	Less snake-like	Snout somewhat pointed. Tail pointed	do	do	do	do	Very much reduced. In the retina sensitive layler is a mass of loose degenerate nuclei	Present but only on the head

From the above statement the following inference can be made. Of the two species of Symbranchidæ, it will be noticed that *Amphipnous fossorius* sp. nov. is more adapted for a burrowing habit. Of the two Gobioidæ *Tænioides cirratus* (Blyth) is more advanced in this direction.

Summary

1. Detailed descriptions are given of the morphology and bionomics of the following species: *Amphipnous fossorius* sp. nov., *Symbranchus bengalensis* (McClleland), *Tænioides cirratus* (Blyth), and *Trypauchen vagina* (Bloch and Schneider). It is pointed out that the first one is a typical burrower.

2. Adaptations for a burrowing habit such as shape of body, snout, loss of fins, etc., are described and discussed in all the four forms.

3. An account of the eyes in all the four forms is given. The eyes are reduced in size in all and show various degrees of degeneration. The cephalic cutaneous organs in *Tænioides cirratus* (Blyth) and *Trypauchen vagina* (Bl. and Sch.) and the lateral cutaneous organs also of the former are described.

Acknowledgements

This work was carried out in the Marine Biological Laboratory of the Travancore University under the guidance of Dr. C. C. John, formerly Professor of Marine Biology and Fisheries, University of Travancore. I take this opportunity to express my deep sense of gratitude to him for suggesting this problem and helping me throughout with his valuable and constructive criticisms, and also for kindly handing over to me his collections of *Amphipnous fossorius*. My thanks are also due to the University of Travancore for awarding me a scholarship during the tenure of which this work was carried out. I am indebted to Professor R. Gopala Aiyar, Head of the Department of Zoology, Andhra University, for going through the manuscript and suggesting suitable alterations before sending it to the Press.

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Key to Lettering

(Terms used in naming the parts of the retina are taken from *Elements of Comparative Anatomy of Vertebrates*, by R. Wiedersheim, 1886.)

sc.	sclerotic.	*i.n.l.*	inner nuclear layer.
ch.	choroid.	*i.g.l.*	inner granular layer.
rt.	retina.	*g.c.*	layer of nerve cells.
pg.	pigment.	*i.l.m.*	internal limiting membrane.
r.c.	layer of rods and cones.	*ls.*	lens.
e.l.m.	external limiting membrane.	*o.c.*	optic cup.
o.n.l.	outer nuclear layer.	*op.n.*	optic nerve.
o.g.l.	outer granular layer.	*cor.*	corpuscles.

STUDIES ON FOLIAR SCLEREIDS IN DICOTYLEDONS

V. Structure and Development of the Terminal Sclereids in the Leaf of *Memecylon heyneanum* Benth.

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(M. R. Science Institute, Gujarat College, Ahmedabad)

Received May 5, 1951

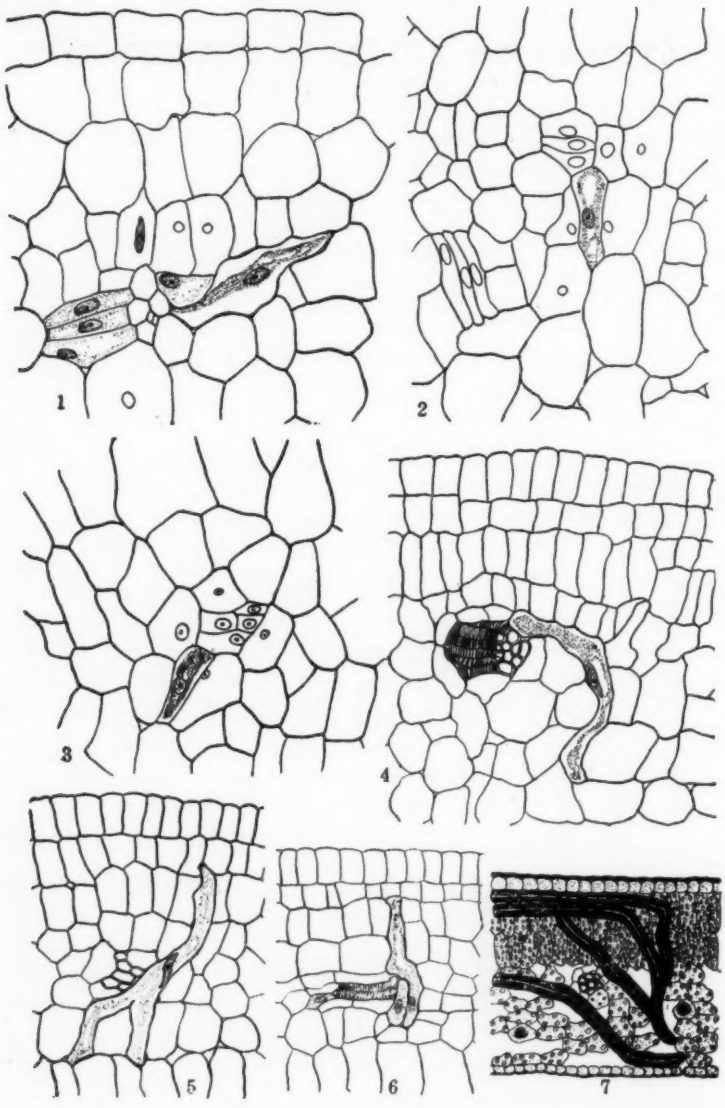
(Communicated by Prof. P. Maheshwari, F.A.SC.)

Recent ontogenetic studies by Rao (1951 *a*, 1951 *b*) have revealed that the apparent terminal position of some of the sclereids as seen in the mature leaf is due to the close juxtaposition of sclereids with vein-endings. Recently, the finding of terminal and sub-terminal sclereids in many species of *Mouriria* (Foster, 1946) and *Memecylon* (Foster, 1946; Subramanyam and Rao, 1949; and Rao, 1950) prompted me to make detailed observations on the origin, structure and development of sclereids in the leaf of *Memecylon heyneanum* in view of its close systematic relationship with the genus *Mouriria*.

Leaf material of *Memecylon heyneanum* a medium sized tree was collected at the Government Botanic Gardens, Bangalore. Vegetative buds as well as small portions of immature and matured leaves were fixed in formalin-acetic alcohol. The fixed material was dehydrated and embedded in paraffin according to customary methods. Microtome sections were stained by Foster's method (1934) and whole mounts of the sclereids were made as outlined by Subramanyam and Rao (1949).

Distribution of Sclereids in the Mature Lamina

The leaf structure in *Memecylon heyneanum* is bifacial. The mature lamina has two-layers of well developed palisade cells and a moderately lacunate spongy parenchyma. The epidermis has a well developed cuticle. As described by Subramanyam and Rao (1949), the most salient feature of the lamina is the presence of elongated ophiuroid or filiform sclereids (Fig. 7) which traverse the leaf in all directions and occasionally exhibit some forking. The adaxial side of the cleared lamina shows sclereids at the ends of veinlets, whereas on the abaxial side the sclereids run in criss-cross manner and sometimes the sclereid ends are found near the stomatal crypts. Another noteworthy feature is that near the margin of the leaf the sclereids are usually disposed in groups of 5 to 12 and give a strand-like



FIGS. 1–7.—Figs 1–3. Transections of young laminæ showing the terminal and sub-terminal dispositions of sclereid initials (× 600). Figs. 4–6. More advanced stages. Note the branching and the proximity of the sclereid near the veinlets (× 300). Fig. 7. Transection of lamina showing mature sclereids (× 150).

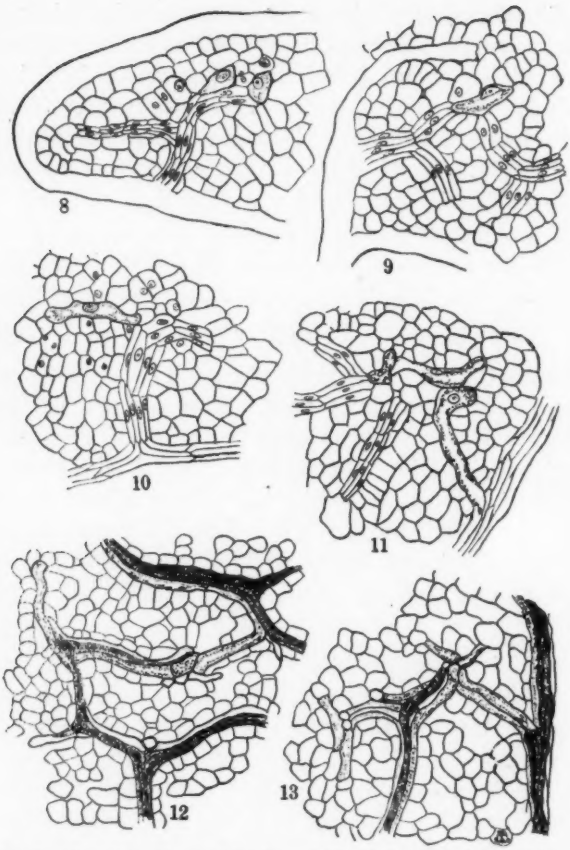
appearance. Small tracheary veins are free from a sclerenchymatous sheath, but the larger veins are sourrounded by well developed sclerenchymatous cells. All the veinlets show at their tips of group of 2 to 5 sclereids. A comparative study of macerations and transections revealed that the sclereids are very much elongated in forms. Mostly they are ophiuroid or filiform with uniform breadth, smooth outer surface and narrow lumen. Wherever there is a forking this takes place at the growing tip resulting in "T" or "Y" shaped forms. Sclereids of this type have been encountered in many species of *Memecylon* (Subramanyam and Rao, 1949; Rao, 1950). The cell wall is homogeneous and has pit canals of either straight or oblique disposition.

Ontogeny

In early stages the young unexpanded lamina consists of closely packed parenchymatous cells. Sometimes small air spaces are distinguishable in the abaxial part and procambial cells are seen in active stages of division. Transverse and paradermal sections of the young lamina revealed that as in *Mouriria huberi* (Foster, 1947) the sclereid initials are differentiated at the tips of the procambial strands (Figs. 1–3, 6, 8–10). At the time of their initiation, the lamina shows only a slight tissue differentiation. The epidermal cells become charged with tannin and palisade tissue is about to be differentiated.

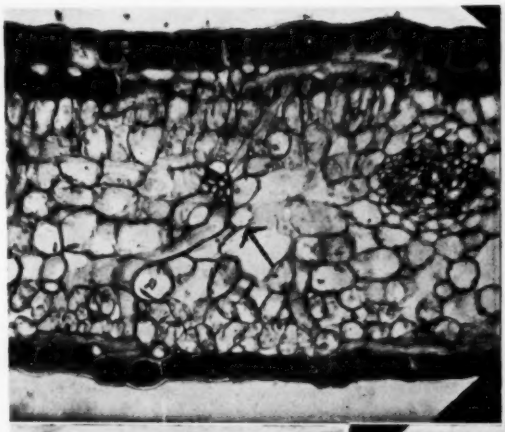
The sclereid initials are distinguishable from the adjacent cells by their shape, size and enlarged nucleus. They are irregular, parenchymatous forms exhibiting a strong tendency to acquire a tube-like shape with a conspicuous pear-shaped nucleus and dense protoplasm. Many efforts were made to recognise the initial cells at a still earlier stage, but this was found to be very difficult as all cells adjacent to the procambial cells were more or less identical. The sclereid initiation coincides with the formation of air-spaces especially in the abaxial region of the lamina.

The sclereid initials soon become tube-like and have uniform width and thick cell wall. They acquire a bipolar growth as reported in *Mouriria* (Foster, 1947), *Diospyros* (Rao, 1951 *a*), and *Olea* (Rao and Kulkarni, 1952) but the growth is confined to the tips only (Figs. 4–5, 10–11). The growth is inter-cellular and multi-directional. The majority of the sclereids exhibit a great tendency to move towards the epidermal layers. It is not uncommon to see them running in the form of a regular tube beneath the epidermal cells to a considerable length. They are abundant in the intra-epidermal or sub-epidermal regions of the lamina, and sometimes exhibit branching (Fig. 5). At this stage the mesophyll shows a good deal of tissue maturation with abundant chloroplasts and tannin in the cells.



FIGS. 8–13. *Paradermal sections of laminæ.*—Fig. 8. Sclereid initial cells showing terminal and sub-terminal disposition at the end of the veinlet. Note their irregular shape and conspicuous nucleus (× 300). Fig. 9. Young sclereid initial in contact with ends of two veinlets (× 300). Fig. 10. Young tube-like sclereid initial (× 300). Fig. 11. Young terminal and diffuse sclereid initials (× 300). Figs. 12–13. Paradermal sections of immature laminæ illustrating terminal disposition of sclereids. Note their appearance in groups at the end of the tracheary vein and a strong tendency to incurve towards the veins before diffusing (× 150).

The tube-like sclereids have a smooth cell wall free from spicules. The protoplasm seems to be uniformly distributed and the pear-shaped nucleus may occupy different positions in the growing cell. A careful examination of paradermal and transections indicated the intimate association of sclereids with the smaller veins of the mesophyll (Figs. 12–19). The sclereids show two trends. Either they follow the veinlets before diffusing elsewhere, or run independently in criss-cross manner. Mostly they are



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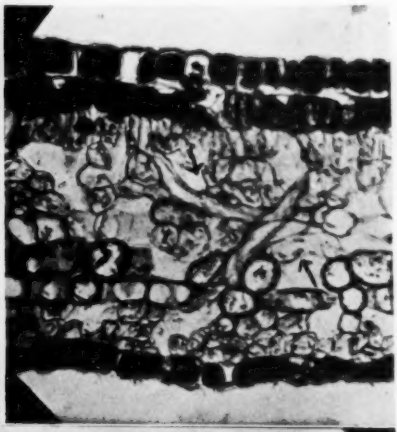

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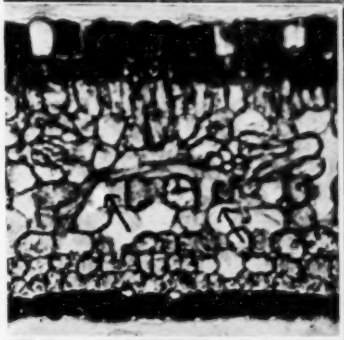

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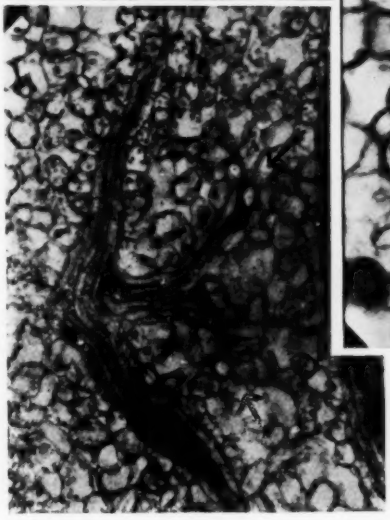

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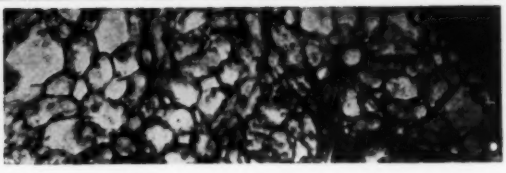

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disposed in strands near the margin of leaves. Near the lower epidermis, it is not unusual to find the fusoid tip of the elongated sclereid cell near the stomata.

At maturity the sclereid initials undergo secondary thickening and possess a median lumen of uniform width. The cell wall is homogeneous and free from any spicules. The presence of straight or oblique pits in the cell wall could be made out. The lumen is free from protoplast and sometimes either obliterated or of irregular width.

Conclusion and Summary

The sclereid initials of *Memecylon heyneanum* show a terminal or subterminal relationship with the procambial strands as reported in the allied genus *Mouriria huberi* (Foster, 1947). The irregularly shaped initials soon acquire a tube-like shape and show local apical growth as reported in *Mouriria huberi*. Sometimes they branch, especially beneath the epidermis.

The occurrence of sclereid initials near the differentiating procambial strands and their strictly terminal or sub-terminal relationship confirms the close relationship between *Mouriria* and *Memecylon* under the sub-tribe *Mouririees* under the family *Melastomaceæ*,—a fact which was recognised by Van Tieghem as early as 1891.

I wish to thank Prof. P. Maheshwari for going through the manuscript. My thanks are also due to Dr. M. J. Thirumalachar for keen interest in my work.

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Explanation of Plate

Figs. 14–17.—Transections of expanded immature leaves showing the contact between young ophiuroid sclereids and the immature veinlets. Figs. 14–16, (× 225); Fig. 17, (× 450).

Figs. 18–19. Paradermal sections of immature leaves. Note the terminal disposition of groups of sclereids at the end of veinlets (× 225).

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INDEX TO VOL. XXXIV—(B)

AUTHORS' INDEX

Bandyopadhyay, K. S. .. *See* Khanna and Bandyopadhyay.

Banerji, I. .. Pollen and embryo-sac of two euphorbiaceæ, 172.

Baxi, A. J., Samarth, Kumud D. and Venkataraman, P. R. The effect of a single injection of thyroxin on the protein and nucleic acids of the mouse liver and the protein of the mouse carcass, 258.

Bhatnagar, V. B. .. *See* Choudhri and Bhatnagar.

Choudhri, R. S. and Bhatnagar, V. B. .. Some aspects of regeneration in leaf cuttings of tomato, 43.

De, N. N. .. *See* Rao and others.

Desai, S. V. and Subbiah, B. V. Nitrification in relation to cation absorption by plants, 73.

Dnyansagar, V. R. .. Embryological studies in the leguminosæ, III, 188.

Gates, G. E. .. Regeneration in an Indian earthworm, *Perionyx millardi* Stephenson, 115.

Kapoor, S. L. .. Importance of manuring in potato crop raised from cut pieces, 99.

Kasturirangan, L. R. .. Placentation in the sea-snake, *Enhydrina schistosa* (Daudin), 1.

Khanna, K. L. and Bandyopadhyay, K. S. Studies in sampling technique, 210.

Krishnamoorthi, B. .. Studies on the osmotic properties of the eggs and larvæ of a brackish-water polychæte, *Marphysa gravelyi* Southern, 199.

Krishnamurti, K. and Soman, S. V. Studies in the adsorption of bacteria, I, 81.

Manna, G. K. .. *See* Ray-Chaudhri and Manna.

Moorthy, B. Rama .. Minor elements in soil and their effect on forage and feeding cattle with special reference to their significance as traces of evolution of some complex organic structures: as determined by application of Goldschmidt's Law, 46.

Müntzing, Arne .. Genetics in relation to plant breeding, 227.

Nayar, K. Krishnan .. Some burrowing fishes from Travancore, 311.

Oke, J. G. .. Flowering habits of *Dichanthium annulatum* Stapf., *Dichanthium caricosum* A. Camus and *Bothriochloa intermedia* (Br.) A. Camus, 165.

Pai, K. V. Srinivasa	..	*See* Ram and Pai.
Pavgi, M. S., Pithawala, H. R., Savur, G. R. and Sreenivasan, A.		Polygalacturonase activity of *Aspergilli*, 33.
Pithawala, H. R.	..	*See* Pavgi and others.
Ramakrishnan, T. S.	..	Additions to fungi of Madras, X, XI, 63, 157.
Ramaswamy, A. S.	..	*See* Rao and others.
Ram, C. S. Venkata and Pai, K. V. Srinivasa		Fertilization value of tank silts, 109.
Rao, R. Rama, Ramaswamy, A. S. and De, N. N.		Immunological study in Avian malaria (*P. gallinaceum*).
Rao, T. Ananda	..	Studies on foliar sclereids in dicotyledons, I, V, 92, 329.
Rao, T. S. Satyanarayana		On a new caligid parasite, *Gloiopotes zeugopteri*, sp. nov., from *Xiphias zeugopteri*, Lawson's Bay, Waltair, 248.
Ray-Chaudhri, S. P. and Manna, G. K.		Chromosome evolution in wild populations of acrididæ, I, 55.
Samarth, Kumud D.	..	*See* Baxi and others.
Savur, G. R.	..	*See* Pavgi and others.
Sitaraman, N. L.	..	Studies on the common bamboo-borer, *Dinoderus ocellaris* Steph., III, 148.
Sivaramakrishnan, V. R.		Studies on early development and regeneration in some Indian marine sponges, 273.
Soman, S. V.	..	*See* Krishnamurti and Soman.
Sreenivasan, A.	..	*See* Pavgi and others.
Sreenivasan, A. and Wandrekar, S. D.		Excretion of vitamin C during germination of leguminous seeds, 267.
Subbiah, B. V.	..	*See* Desai and Subbiah.
Subramaniam, M. K.	..	Nature and nurture in yeasts, 220.
Venkataraman, P. R.	..	*See* Baxi and others.
Venkatesh, C. S.	..	The inflorescence and flowers of *Dichrostachys cinerea*, W. and A., 183.
Venkatram, C. S.	..	A photographic method for determining apical growth in fungal hyphæ, 256.

TITLE INDEX

Acrididæ, wild populations, chromosome evolution, I (Ray-Chaudhri and Manna), 55.

Aspergilli, polygalacturonase activity (Pavgi and others), 33.

Avian malaria (*P. gallinaceum*), immunological study, by the anaphylactic reaction (Rao and others), 242.

Caligid parasite, *Gloiopotes zeugopteri* sp. nov., new, from *Xiphias zeugopteri*, Lawson's Bay, Waltair (Rao), 248.

Dichanthium annulatum Stapf., *Dichanthium caricosum* A. Camus and *Bothriochola intermedia* (Br.) A. Camus, flowering habits of (Oke), 165.

Dichrostachys cinera W. and A., the inflorescence and flowers (Venkatesh), 183.

Enhydrina schistosa (Daudin), sea-snake, placentation (Kasturirangan), 1.

Euphorbiaceæ, two, pollen and embryo-sac (Banerji), 172.

Fishes, burrowing, some, from Travancore (Nayar), 311.

Fungal hyphæ, apical growth, a photographic method for determining (Venkatram), 256.

Fungi of Madras, additions, X, XI (Ramakrishnan), 63, 157.

Genetics in relation to plant breeding (Müntzing), 227.

Leguminosæ, embryological studies, III (Dnyansagar), 188.

Marphysa gravelyi Southern, brackish-water polychæte, studies on the osmotic properties of the eggs and larvæ (Krishnamoorthi), 199.

Protein and nucleic acids of the mouse liver and the protein of the mouse carcass, the effect of a single injection of thyroxin (Baxi and others), 258.

Sampling technique, studies, V (Khanna and Bandyopadhyay), 210.

Sclereids, foliar, in dicotyledons, studies, V (Rao), 329.

Soil, minor elements in, and their effect on forage and feeding cattle with special reference to their significance as tracers of evolution of some complex organic structures: as determined by application of Goldschmidt's Law (Moorthy), 46.

Sponges, Indian marine, some, studies on early development and regeneration (Sivaramakrishnan), 273.

Tomato, leaf cuttings, some aspects of regeneration (Choudhri and Bhatnagar), 43.

Vitamin C, excretion, during germination of leguminous seeds (Sreenivasan and Wandrekar), 267.

Yeasts, nature and nurture (Subramaniam), 220.